PLANT GROWTH, METAL UPTAKE, AND SOIL INTERACTIONS OF THE HEAVY METAL HYPERACCUMULATOR *PELARGONIUM* SP. 'FRENSHAM' AND THE HEAVY METAL EXCLUDERS *SILENE VULGARIS* AND *AGROSTIS CAPILLARIS* GROWN IN SMELTER-IMPACTED SOILS

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By
Jennifer Lynn Bentz

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ABSTRACT

Industrial mining and processing of zinc and copper ore in Flin, Flon MB/ Creighton, SK by the Hudson Bay Mining and Smelting Co. Limited (HBMS; a division of Hudbay Minerals Inc.) has led to the enrichment of the surface soils with heavy metals such as copper (Cu), zinc (Zn), and cadmium (Cd) and soil acidification from sulfur dioxide. In addition to forest dieback resulting from heavy metal contamination and soil acidification, forest fires and timber harvests have resulted in considerable loss of native forest. As a soil amendment for revegetation, the community-lead Flin Flon/ Creighton *Green Project* has been adding dolostone to the surface of the soil. This project has resulted in varying and unexplained levels of success with significant re-growth of trembling aspen (*Populus tremuloides*) and white birch (*Betula papyifera*) at some sites, with a lack of response at others.

This study was conducted to examine the effects of the dolostone used in Flin Flon, MB/ Creighton, SK on the soil properties, metal availability, and plant growth to understand the variable response of liming at the site. In addition, metal uptake, low molecular weight organic acids in the soil, changes in pH, and changes in available metals were analyzed after the growth of a metal hyperaccumulating plant *Pelargonium* sp. 'Frensham' and two metal excluding plants *Silene vulgaris* and *Agrostis capillaris* to determine the interactions of metal-tolerant plants with the soils after liming. These plants were chosen to examine the effects of the different mechanisms of metal tolerance on metal uptake, mobility, and bioavailability in contaminated soils after liming. Limed and non-limed soils from a responsive site at Knight North (KN) and an non-responsive site at Second Valley North (SVN) were collected from Flin Flon, MB/ Creighton, SK, along with a reference soil from an undisturbed boreal forest east of the smelter, for a growth trial.

Liming had a positive effect on the soil, increasing the pH, decreasing the available fraction of metals, and increasing plant growth. On the SVN site, the pH only increased from 4.01 to 4.25, while the available NO_3^- and exchangeable calcium (Ca^{2+}) and (Mg^{2+}) remained low, indicating the dolostone had not dissolved or had leached out. Subsequently, the available metal fraction decreased, however Zn^{2+} remained at 296.3 mg kg^{-1} contributing to the toxicity of the soil. On the KN site, the initial NO_3^- was the highest for all the soils, and the dolostone increased the pH from 3.36 to 5.10 while increasing the exchangeable Ca^{2+} and

Mg^{2+} above the reference soil. The increase in pH caused by the dolostone lead to a substantial decrease in the available metal fraction on this soil and increase in plant biomass. Plant growth increased from the KN non-limed < SVN non-limed < SVN limed < KN non-limed < reference soil. The lower response to the lime at the SVN site was attributed to the lack of dolostone dissolution on site and/or leaching of Ca and Mg, the dense sod matt of *A. capillaris* inhibiting germination of other plant species, as well as the low nutrient status of the soil.

Overall plant growth had a positive effect on the soils, especially the metal-excluders *S. vulgaris* and *A. capillaris* in the SVN soil. Both plants significantly increased the pH of the soil and decreased the available fraction of Al^{3+} in the non-limed soil, and Cu^{2+} and Al^{3+} in the limed soil. *Pelargonium* sp. 'Frensham' did not significantly change the pH of the soil, however the plants accumulated high concentrations of Cu, Zn, Cd and Al in the plant tissues; and *P.* sp. 'Frensham' decreased the available fractions of Cu, Zn, and Al in the SVN limed soil, and Zn in the KN non-limed soil. For the three plant species used, liming decreased the concentration of heavy metals in the shoots. Where plants established in the non-limed soils, the concentration of heavy metals in the shoots was larger than in the reference soil and limed soils, except for *P.* sp. 'Frensham' with Al. On the SVN soils, liming increased the concentration of Al in the shoots near the threshold of hyperaccumulation at 1000 mg kg^{-1} dry weight. For the most part, higher shoot concentrations of heavy metals in the non-limed soils indicate that lime should be used to decrease the uptake of heavy metals into the aboveground biomass, even if vegetation is present in the soil without lime, to control the transfer of metals to other components of the ecosystem.

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AEM	anion exchange membrane
CECe	effective cation exchange capacity
DEE	diethyl ether
FID	flame ionization detector
GC	gas chromatography
HBMS	Hudson Bay Mining and Smelting
KN	Knight North Site
LMWOA	low molecular weight organic acids
MP-AES	microwave plasma atomic emission spectroscopy
M-TBSTFA	*N*-[*tert*-butyldimethylsilyl]-*N*-methyl-trifluoroacetamide
SPE	solid phase extraction
SVN	Second Valley North site
t-BDMS	*tert*-butyldimethylsilyl
TEA	triethylamine

1 INTRODUCTION

Industrial processes, such as mining and smelting, have led to extensive areas being impacted by soil acidification from sulfur dioxide and contaminated with multiple heavy metals. Unlike organic contaminants, heavy metals cannot be degraded in the environment and can pose a persistent threat to higher plants and organisms. By directly reducing or inhibiting physiological and biochemical processes essential for life, heavy metals can affect growth, photosynthesis, respiration, and cellular organelle function (Garbisu and Alkorta, 2001). Many of these heavily disturbed sites also suffer from severe forest dieback and a loss of biological diversity leaving the soils susceptible to wind and water erosion. Remediation of soils heavily contaminated with metals is often difficult and traditionally involves excavation and treatment, or burial of the polluted soil, both of which are expensive and environmentally destructive (Marques et al., 2009). For large, heavily contaminated sites, *in situ* immobilization using soil amendments to chemically transform, complex, or precipitate metals into a form that is less toxic, mobile, or bioavailable is a common technique in site revegetation or rehabilitation (Marques et al., 2009).

Hudson Bay Mining and Smelting, Co. Ltd. (HBMS; a division of Hudbay Minerals Inc.) has been mining and processing zinc and copper ore in Flin Flon, MB/Creighton, SK since 1930. The Boreal ecosystem surrounding the mine/refinery complex has suffered considerable loss of the native forest as a combined result of forest fires and timber harvests followed by metal contamination and soil acidification from the smelting of sulfide ores that has stalled recovery. Despite technological improvements to the smelter to reduce atmospheric emissions and improve air quality, the area has remained relatively barren, containing only metal tolerant grasses such as colonial bent grass (*Agrostis capillaris*) and relict boreal species that are stunted and unable to reproduce naturally (Winterhalder, 2003). In an effort to revegetate the barren area around the smelter—and on the advice of Professor Keith Winterhalder, who was involved in efforts to revegetate a similar site in Sudbury, ON (Winterhalder, 2001)—a community led initiative known as the Flin Flon/Creighton *Green Project* began applying crushed (pea-sized) dolostone [$CaMg(CO_3)_2$] to the surface of the soil in 2000. To date, the project has had varying levels of success, with significant re-growth

of trembling aspen (*Populus tremuloides)* and white birch (*Betula papyifera)* at some sites, and a lack of response at others. In addition to the inconsistent vegetative response to the dolostone applications, the species diversity remains low on the vegetated sites, which is problematic for succession back to a healthy, functioning ecosystem.

Applications of lime (or, in this case, dolostone) to the soil act to neutralize acidity by reacting with water to create hydroxyl (OH^-) ions that can combine with, and neutralize, free or exchangeable hydrogen ions (H^+). In return, pH-induced immobilization of metals in the soil occurs from an increase in pH-dependent cation adsorption sites and the formation of metal hydroxides. These metal hydroxides bind to adsorption sites with a higher affinity than the metal cations themselves or precipitate out of solution (Bolan and Duraisamy, 2003). The effectiveness of the lime in neutralizing acidity and decreasing the toxicity of the metals present in the soil is controlled by the quantity and quality of the lime added, soil texture, soil cation exchange capacity, and metal concentration(s). However, as plants grow in the amended soil, they release a number of compounds into the rhizosphere that may mobilize the adsorbed/precipitated metals increasing their availability.

A better understanding of the soil-root chemistry of plants with respect to heavy metal uptake, bioavailability, and mobility is crucial for developing an effective and sustainable revegetation strategy for the HBMS site in Flin Flon, MB/Creighton, SK (as well as other mine sites in the Boreal forest). Characterizing the plant derived low molecular weight organic acids of metal hyperaccumulating species and metal excluding species also may assist in identifying the reactions of metal accumulation and exclusion in the soil, and be used as a possible screen for selecting appropriate plant species for phytoremediation projects. Moreover, given the widespread use of limestone for phytostabilization of large, heavily contaminated sites, characterizing the effect of lime on metal mobility, metal toxicity, and root exudation on sites with wide ranging metal concentrations may aid in understanding the variable response seen in Creighton/Flin Flon by the *Green Project*. Thus, the specific objectives (and hypotheses) of this study are to:

1. Examine how soil properties, metal availability, and plant growth of a metal hyperaccumulating plant and two metal excluding plants were affected by additions of dolostone to lime responsive and non-responsive soils.

Hypothesis: Differences in soil chemical properties and heavy metal contamination are affecting the response of dolostone in the soil in alleviating metal toxicity and, in turn, the soil chemistry, plant interactions with the soil, and metal uptake by the plants.

2a. Determine the metal concentrations in the roots and shoots of a known metal hyperaccumulator and two known metal excluders grown in multi-metal contaminated soils from the lime responsive and non-responsive sites.

2b. Characterize the type and quantity of low molecular weight organic acids released by the roots of a metal hyperaccumulator and two metal-excluder plants grown in multi-metal contaminated soils from the lime responsive and non-responsive sites, and the subsequent changes in pH, total metals, and available metals in the soil.

Hypothesis: Hyperaccumulators and excluders interact differently with heavy metals in the soil, and root-derived low molecular weight organic acids facilitate exclusion of metal uptake into plant roots.

2 LITERATURE REVIEW

Revegetation strategies should address the issue of sustainability while reducing the ecological risks of metal transfer from the soil to the plant and decreasing the mobility of metals in the environment after vegetative establishment (Yang et al., 2010). Moreover, plant-induced modification of the soil in the rhizosphere can alter organic acid concentration profiles, pH, redox conditions, and microbial community diversity and activity, which in turn can affect metal speciation and bioavailability. The direction and extent of these changes in the rhizosphere is species and site dependent, which reflect plant nutrient acquisition strategies, root architecture, and soil properties (Kidd et al., 2009). Therefore, understanding how different plant species can affect the behavior of metals in the soil will aid in developing effective long-term revegetation strategies that limit metal mobility and uptake at the landscape-scale.

2.1 Chemistry and Long-term Stability of Revegetated Sites

Whereas liming amendments have proven successful for revegetation projects like the one in Sudbury, ON, the long-term stability of these sites is in question—as liming affects the bioavailability of the heavy metals and not the total concentration in the soil. Over time, immobilized heavy metals may recycle into plant available forms through natural or plant induced weathering of soil particles, the breakdown of organic-metal complexes, or through re-acidification of the soil (Bolan and Duraisamy, 2003). Moreover, nutrient and metal cations exist in a dynamic equilibrium in the soil where leaching or plant uptake can force the exchangeable metal fraction back into the soil solution. In a two-year study analyzing the effect of vegetation on metal mobility on an abandoned Cu mine in China, Yang et al. (2010) found that DTPA-extractable Zn and Pb increased significantly over time in the vegetated plots, while Cu remained the same. Yang et al. (2010) also found increasing concentrations of Zn, Pb, and Cu in the root and shoot tissues of all the plants in the study, with Zn concentrations increasing the most.

Contrary to Yang's results, McGrath et al. (1997) found the concentration of mobile Zn to decrease in both rhizosphere and non-rhizosphere soils in a growth chamber study with the

hyperaccumulator *Thlaspi caerulescens*. Despite accumulating significant amounts of Zn in the plant shoot tissues, the decrease in Zn concentrations in the soils accounted for only 10% of the mobile Zn, suggesting that the plants were accessing the non-mobile fraction. In comparison with the non-accumulator *Thlaspi ochroleucum*, both plant species caused similar decreases in the pH of the rhizosphere, indicating that acidification of the soil was not the main mechanism for Zn accumulation, and that other species-specific changes in the rhizosphere were responsible for the enhanced Zn uptake by the hyperaccumulator (McGrath et al., 1997).

In another two-year study growing the graminaceous plant *Festuca rubra* in metal contaminated sediment, Panfili et al. (2005) found that after an initial increase in Zn bioavailability in the soil, the plants formed black precipitates on the roots preventing the translocation of Zn to the shoots. These precipitates inhibited Zn accumulation in the aboveground biomass of the plants. Lanson et al. (2008) later identified these precipitates as Zn phyllomanganate nanoparticles formed by the plant to reduce metal toxicity.

2.2 Heavy Metals and Plants

With conflicting studies on the effect plants have on metal availability and uptake, no clear pattern exists as to whether plants enhance or deplete the mobile fraction of metals in the soil, or the mechanisms plants use to accumulate metals. Different plant species appear to have different effects on the behavior of certain heavy metals in the soil, which are not directly related to pH-induced changes in the rhizosphere. Plants release a number of different compounds from the roots as a response to stress, nutrient acquisition, and protection from toxic substances. The compounds released by rhizodeposition include: root exudates (organic acids), secretions (ectoenzymes), plant mucilage (carbohydrates), mucigel (cellular debris), and root lysates (phenolics) (Kidd et al., 2009). Environmental conditions, stress, nutrient deficiencies, soil properties, and the presence of toxins can alter the composition of root exudates from plants and, in turn, affect the influence these root exudates have on the soil chemistry (Badri and Vivanco, 2009). Some plants are known to be excluders of one or more heavy metals; examples include *Agrostis capillaris* (Cu, Zn, Cd, and Pb), *Festuca rubra* (Zn), and *Lupinus albus* (Cu). On the other hand, only about 400 plant species—including *Salix viminalis* (Cu, Zn, and Cd), *Thlaspi caerulescens* (Zn and Cd),

and some *Pelargonium* species (Cu, Zn, Cd, Pb, Ni, and B) (Dan et al., 2000; Environment Canada, 2003; Kidd et al., 2009) have been shown to accumulate/hyperaccumalate heavy metals.

2.3 The Development of Metal Hyperaccumulation and Tolerance in Plants

Heavy metals occur in soils from a variety of natural and anthropogenic sources, most common of which are serpentine soils derived from ultramafic rocks; i.e., rocks high in Ni, Cr, and Co (Reeves, 2006). Other types of natural metalliferous soils include calamine soils (high in Pb and Zn, and may contain Cd, As, and Cu), such as those found in the Olkusz Ore-bearing region of Poland; and the Cu/Co-rich soils found in the Democratic Republic of Congo (Reeves, 2006). These metal rich soils vary widely in physical and chemical characteristics, as well as in the types and concentrations of heavy metals present. While some metalliferous soils are toxic to all vegetation, most of these soils contain a unique and characteristic flora of metal tolerant plants (Baumbach, 2012; Reeves, 2006). Based on their interaction with a metal, which is controlled by plant species and genotype, the vegetation present on these metal-enriched soils can be divided into three groups: excluders, indicators, and accumulators (Tlustos et al., 2006).

Heavy metal excluders inhibit the uptake of metals into the plant or the translocation of metals to the shoots of the plant. Most excluders are monocotyledon grasses. Heavy metal indicators generally contain heavy metal concentrations that linearly correlate to available metal concentrations in the soil solution. Most indicators are agricultural plants such as wheat, oats, and maize. Lastly, heavy metal accumulators contain higher concentrations of trace elements in the shoot tissues of the plant, relative to the available metal concentrations in the soil. Some extreme accumulators can survive while taking up very high metal concentrations in the plant tissues, and are defined as hyperaccumulators (Tlustos et al., 2006). The threshold metal concentration (on a dry weight basis) that defines a hyperaccumulator depends on the heavy metal in question; e.g., Cd (>100 mg kg^{-1}); Ni, Pb and Cu (>1,000 mg kg^{-1}); and Zn (>10,000 mg kg^{-1}) (Baker and Brooks, 1989). In general, most hyperaccumulators are dicotyledonous plants.

Of all the hyperaccumulators, accumulators, and excluders identified almost all are Ni tolerant, possibly due to the large extent of serpentine soils found across the world (Reeves,

2006). Uptake of Zn and Cd usually occur together, while Co and Cu are taken up simultaneously due to the phytochemistry of the metals within plants and the presence of these metals together in the environment (Brooks, 1998). However, true hyperaccumulation of Pb, and even Cu, is rare (Reeves, 2006). Lead is usually found in low concentrations in the environment, even on contaminated sites, and is relatively immobile in soils and within plants. Copper uptake is usually tightly controlled within plants, even for hyperaccumulators, and concentrations above 100 mg kg^{-1} dry weight are uncommon.

According to Tlustos et al. (2006), aside from the plant species and genetic aspects of metal tolerance, a number of other factors control the uptake and accumulation, or exclusion of metals in plants. These factors include the mobility of the element within the plant, the concentration of elements in the soil, the availability and mobility of metals in the soil, and the pH, cation exchange capacity, clay content, and organic matter content of the soil. Moreover, for metal excluders, tolerance to a specific contaminant has been linked to the selective pressure for certain genes within a plant species, invoked by toxic concentrations of the contaminant in the environment (Schat and Vooijs, 1997). Thus, varying levels of tolerance to a number of heavy metals have been recorded for the same plant species (Schat and Vooijs, 1997).

In order to aid in characterizing the poorly understood reactions of metal hyperaccumulators and excluders with heavy metals in the soil, three plant species were chosen for their known ability to tolerate Cu, Zn, and Cd as these are the main metals of concern at the HBMS site in Flin Flon, MB/Creighton, SK. These concerns arise from the high concentrations of Cu (23 to 12,769 mg kg^{-1}) and Zn (231 to 15,716 mg kg^{-1}) found at the site (Bentz and Farrell, unpublished data, 2012); Cd, on the other hand, was found at much lower concentrations (≤113 mg kg^{-1}), but is toxic in low doses (Pahlsson, 1989). The three plant species chosen for this study were: *Pelargonium* sp. 'Frensham' which is a lemon-scented geranium capable of hyperaccumulating/accumulating Cu, Zn, Cd, Pb, Ni, and B (Saxena and KrishnaRaj, 1999; KrishnaRaj et al., 2000); *Silene vulgaris,* which is a perennial herb, also known as bladder campion, that is capable of excluding Cu, Zn, and Cd for certain ecotypes (Schat and Vooijs, 1997); and *Agrostis capillaris,* or colonial bentgrass, which also contains ecotypes able to exclude Cu, Zn, Cd, and Pb (Dahmni-Muller et al., 2000).

2.3.1 *Silene vulgaris*

Schat and Vooijs (1997) analyzed the genetics of co-tolerance to multiple heavy metals for different ecotypes of *S. vulgaris* by crossing the different ecotypes of populations growing on different metalliferous sites in Germany. Ecotypes included plants that were slightly tolerant to Cu, Zn, and Cd (collected from Marsberg); plants that were highly tolerant to Cu, Zn, and Cd (collected from Imsbach) and plants that were tolerant to Zn, Cd, Ni, and Co (collected from Blackenrode). Schat and Vooijs (1997) found that Cd, Zn, and Cu tolerance in *S. vulgaris* were under the control of different genes and that the presence of toxic concentrations of metals could "provoke highly specific genetic adaptations" in the plant that resulted in a tolerance to the metals. Moreover, Schat and Vooijs (1997) found that these adaptations might also result in low levels of co-tolerance to other metals with similar biochemical reactivity. The presence of co-tolerance between Zn and Cd was attributed to the regular presence of the two metals in toxic concentrations in mine spoils. However, the non-functional co-tolerance of Zn and Cd in the Cu tolerant Imsbach population (which occurred near a Cu mine) could not be concretely deduced, as Zn and Cd were not enriched in the Imsbach mine soil. Schat and Vooijs (1997) hypothesized that the tolerance to Zn and Cd arose from a historical selection pressure for these metals, as the site was formerly and Zn, Cd and Pb mine, and that the maintenance of these genes over time, despite the lack of selective pressure, is due to the absence of non-tolerant genes in the genetic pool. The Imsbach site is isolated from non-tolerant populations by a dense forest surrounding the mine and likely protected from the reintroduction of non-tolerant alleles for Zn and Cd due to the extreme Cu-toxicity in the soil, in which crosses between the Imsbach population and non-tolerant populations would not survive (Schat and Vooijs, 1997; Baumbach, 2012).

Song et al. (2004) compared the tolerance and uptake of Cu in *S. vulgaris* and *Elsholtzia splendens* on thirty different soils from contaminated sites in the UK, Chile, and China, each with differing concentrations and availabilities of Cu. On all the soils, *S. vulgaris* established and grew well—showing no signs of toxicity-induced stress or changes in shoot biomass. They attributed this to the high tolerance of the plant to Cu; indeed, *S. vulgaris* survived in the most contaminated soil, which contained 8,645 mg kg^{-1} of total Cu and a soil solution concentration of 276 μM soluble Cu. The concentration of Cu in the shoots of *S. vulgaris* ranged from 3 to 262 mg kg^{-1}, which is below the 1,000 mg kg^{-1} threshold for a Cu-

hyperaccumulator (Baker and Brooks, 1989). The concentration of Cu in the roots was significantly higher than the shoots, averaging 11- to 30-times the shoot concentration, which is typical for many metal excluding plants. Song et al. (2004) used regression analysis to determine whether—based on Cu^{2+} activity in *S. vulgaris*—dissolved organic carbon (DOC) and pH significantly impacted root Cu concentrations. They found that, contrary to expectations, increasing the concentration of DOC and increasing the pH of the soil solution had a positive effect on the Cu concentration of the roots. Because Cu was retained on the exchange sites of the DOC, Song et al. (2004) attributed the positive correlation to an increased capacity of the solution to buffer against depletion of Cu^{2+}. The positive correlation with pH was explained as a result of an increase in binding capacity of biotic surfaces, such as root cell walls and membranes, at higher pHs.

The possible mechanisms of Cu tolerance and toxicity for *S. vulgaris* in the Imsbach, Marsberg, and non-tolerant populations were assessed by van Hoof et al. (2001) by comparing the Cu uptake of isolated, inside-out oriented plasma membrane vesicles from the roots of the plants. The most tolerant population (Imsbach) accumulated two to three times the amount of Cu in these vesicles compared to the other two ecotypes when ATP was supplied—but little or no accumulation when ATP was absent. The reliance of the transport system on ATP indicated that Cu transport in *S. vulgaris* is active rather than passive. Moreover, van Hoof et al. (2001) concluded that a significant difference in Cu-efflux between the tolerant and non-tolerant populations provided evidence that the genetic control of Cu tolerance is related to the expression of a Cu efflux transporter. The hyper-tolerance of Imsbach populations of *S. vulgaris* is therefore considered to be a result of enhanced efflux of Cu out of the root cells (van Hoof et al., 2001).

2.3.2 *Agrostis capillaris*

Dahmani-Muller et al. (2000) analyzed the heavy metal tolerance and accumulation capacities of various plant species, including *A. capillaris,* growing near a non-ferrous smelter in northern France. In zones dominated by *A. capillaris,* total heavy metal concentrations in the surface (Ah horizon) soil were 10,200 mg Zn kg^{-1}; 4,030 mg Pb kg^{-1}; 469 mg Cu kg^{-1}; and 42 mg Cd kg^{-1}. The highest metal concentrations measured in the roots of *A. capillaris* growing naturally on site were 2,320 mg Zn kg^{-1}; 471 mg Pb kg^{-1}; 65 mg Cu kg^{-1}; and 37 mg Cd kg^{-1}. Metal concentrations were lower in the leaves of *A. capillaris* than

in the roots, confirming that the metal tolerance of this plant was based on exclusion and limited transport to the shoots. The highest shoot concentrations of the metals were only 117 mg Pb kg^{-1}, 30 mg Cu kg^{-1}, and <20 mg Cd kg^{-1}. For Zn, Dahmani-Muller et al. (2000) measured concentrations that were two-fold less in the shoots than in the roots—with some shoot concentrations being greater than 1,000 mg Zn kg^{-1}, which is high for an excluder plant, but still below the 10,000 mg kg^{-1} threshold of a Zn-hyperaccumulator. They went on to suggest that Zn immobilization in the roots of the *A. capillaris* population used in this study was less efficient than that measured in other studies, and that some Zn was being transported to the leaves by diffusion.

2.3.3 *Pelargonium* sp. 'Frensham'

KrishnaRaj et al. (2000) assessed the tolerance of the lemon-scented geranium *P.* sp. 'Frensham' to Pb by measuring the kinetics of chlorophyll *a* fluorescence to evaluate the activity of the photosystem II apparatus in hydroponically grown plants. They found that the geranium remained photosynthetically active when exposed to a Pb concentration of 7.55 m*M,* applied daily as $Pb(NO_3)_2$ for 14 days. At the same time, *Brassica juncea* and *Helianthus annuus* (known hyperaccumulators) both experienced a permanent reduction in photosynthetic efficiency when exposed to the same Pb concentration. *Pelargonium* sp. 'Frensham' remained metabolically active while accumulating 3,005 mg Pb kg^{-1} dry weight in the shoots and 60,986 mg of Pb kg^{-1} dry weight in the roots. KrishnaRaj et al. (2000) also found that the lemon-scented geranium could accumulate the heavy metals Pb, Cd, and Ni when added as a mixed solution to the growth substrate, indicating that the uptake of one metal ion did not interfere with the uptake of the others. The ability to accumulate multiple heavy metals within the plant tissue is an uncommon trait in hyperaccumulators, and distinguishes *P.* sp. 'Frensham' from other metal accumulating species (KrishnaRaj et al., 2000).

In a similar study, Dan et al. (2000) assessed the ability of *P.* sp. 'Frensham' to accumulate the heavy metals Ni and Cd when grown in perlite and watered with a metal salt solution. The Cd content of the roots and shoots increased significantly as the Cd supply was increased from 250 to 750 mg Cd L^{-1}, yielding 42 and 778 mg Cd kg^{-1} dry weight plant biomass, respectively. At a solution concentration of 1,000 mg Cd L^{-1}, Cd accumulation decreased and signs of metal toxicity (such as chlorosis and senescence) were observed in

mature leaves. For Ni, the concentration in the roots of the plants was highest at solution concentrations of 250 and 1,000 mg Ni L^{-1}, but lower at concentrations of 500 and 750 mg Ni L^{-1}. In contrast, Ni concentrations in the shoots increased with increasing Ni supply ranging from 288 mg Ni kg^{-1} at the lowest Ni supply (250 mg L^{-1}) to 1,195 mg kg^{-1} at the highest (1,000 mg L^{-1}). The results of the study indicate that Cd uptake by the roots of *P.* sp. 'Frensham' involved both active and passive transport, whereas only active transport was involved in Ni uptake (Dan et al. 2000). Saxena and KrishnaRaj (1999) grew *P.* sp. 'Frensham' in a greenhouse and field study in multiple, metal contaminated soils for a three-month period. Aside from Ni, Pb, and Cd, the plants also accumulated other metals such as Cu, Zn, and B in the dense, aboveground foliage.

KrishnaRaj et al. (2000) studied the metal detoxification mechanisms in *P.* sp. 'Frensham' by examining the roots of the plants in a scanning electron microscope coupled with an X-ray microanalyzer. When treated with 7.55 m*M* $Pb(NO_3)_2$, the roots of the scented geranium contained a decreasing gradient of Pb concentrations from the epidermis to the central root axis. They concluded that the observation established evidence for both passive and active transport of metal ions through the root apoplasm to the symplasm. Further analysis of the roots using transmission electron microscopy, lead to the discovery of distinct Pb containing globules in the cell membranes, cells walls, cytoplasm, and vacuoles in the treated plants which was not present in the control plants.

2.4 The Rhizosphere

The rhizosphere is defined as the volume of soil affected by plant roots from the exudation of organic substances, carbon dioxide (CO_2), protons, hormones, and the release of root debris (Uren, 2007). Compared to the bulk soil, this zone is characterized by changes in the chemical, biological, and physical properties of the soil that result from highly dynamic biogeochemical processes occurring within the few millimeters of affected soil (Wenzel et al., 2004). These dynamic processes are typically a function of nutrient and water acquisition strategies, as well as stress-related responses of the plant, that create one of the most diverse habitats on earth in terms of microbial ecology (Hinsinger et al., 2009). Highly heterogeneous in both the spatial and temporal distribution of its attributes, the rhizosphere presents considerable challenges in characterization. Due to this high variability of the

rhizosphere and steep gradients created in the soil, this microenvironment cannot be accurately delineated and varies significantly between plants and ecosystems making it difficult to analyze (Wenzel et al., 2004).

2.5 The Type and Quantity of Root Exudates

The majority of root exudates released by a plant are organic substances derived from photosynthesis and C metabolism such as sugars and polysaccharides, amino acids, organic acids, fatty acids, and enzymes. Yet, despite the considerable literature that exists regarding measuring and analyzing the rhizosphere, the role of these root exudates remains highly speculative (Uren, 2007). This is a result of the short life expectancy of the organic compounds within the rhizosphere environment as they are rapidly degraded or assimilated by microorganisms. Nonetheless, root exudates are classified based on their perceived functional role in the soil: i.e., excretions such as CO_2, bicarbonate ions, protons, and electrons facilitate the internal metabolism of plants; secretions such as mucilage, enzymes, phytosiderophores, and allelochemicals assist external processes; and diffusates such as organic acids, amino acids, water, inorganic ions, and oxygen maintain a non-specific, un-quantified role (Uren, 2007). Although quantification of root diffusates is challenging, and their functional role is unknown, they are thought to be important multi-functional components of the soil-root interface for plants. These low molecular weight diffusates have been implicated in nutrient solubilization, enrichment of microbial activity, the dissolution/precipitation of minerals, changes in pH, and ligand exchange reactions—particularly those involving organic acids and micronutrients or heavy metals (Wenzel et al., 2004). However, whether low molecular weight organic acids (LMWOA) complex heavy metals to prevent uptake or facilitate transport within plants is questionable; and depends on the plant species, metal, metal speciation, and soil parameters.

2.6 Organic Acid Interactions with Heavy Metals and Hyperaccumulation

2.6.1 Copper

Like most micronutrients, Cu is an essential element involved in plant nutrition; therefore, Cu concentrations in the shoots of plants tend to be tightly controlled and consistent, independent of external Cu concentrations (Brooks et al., 1980). Moreover, unlike

Ni, Zn, Al, and Cd, the historical occurrence of Cu-rich soils is limited to the Copper Belt of Zaïre/Zambia in the Democratic Republic of the Congo—thus confining the evolution of Cu-tolerant plant species to this area. The Copper Belt is a large area (22,000 km^2) with a diverse flora population creating an environment amenable to the development of advanced plant families tolerant to Cu (Brooks et al., 1980). The two most common Cu-tolerant species found in this belt belong to the *Labiatae* and *Scrophulariaceae* families (Brooks et al., 1980).

Despite the diversity of Cu-tolerant plant species, true hyperaccumulation of Cu is extremely rare. Moreover, most reported cases of Cu hyperaccumulation have been refuted due to a lack of reproducible results under controlled conditions—with shoot Cu concentrations well below the 1000 mg kg^{-1} dry weight threshold (Faucon et al., 2012). It is suspected that Cu-rich dust adsorbs to leaf surfaces in the field (Küpper et al., 2009; Faucon et al., 2012) or that, under laboratory conditions, some physiochemical parameter that affects Cu uptake (e.g., pH, competition with other metals, or interactions with microorganisms) may be deficient. Nonetheless, one of the only true Cu hyperaccumulators is the amphibious water plant *Crassula helmsii*, which was reported by Küpper et al. (2009) to contain 9000 mg Cu kg^{-1} dry shoot weight. However, *C. helmsii* is not a terrestrial plant, and the shoot cells are in contact with dissolved Cu in the water bypassing the uptake, translocation, and storage mechanisms required for terrestrial hyperaccumulators (Küpper et al., 2009). Compared to Zn, Cd, and Al, little information exists on the uptake of Cu for terrestrial hyperaccumulators.

On the other hand, most of the cuprophytes studied in South Central Africa are excluders. Hydroponic studies with *Haumaniastrum katangense*, *Oenothera picensis, Imperata condensate,* and *Crepidorhopalon perennis* for example, have consistently found shoot Cu concentrations lower than 50 mg kg^{-1} shoot dry weight irrespective of the Cu concentration in solution (Faucon et al., 2012; Meier et al., 2012; Peng et al., 2012). As with other excluders, Meier et al. (2012) found high exudation rates of LMWOA for *I. condensate*, *L. albus,* and *O. picensis* under Cu stress. For *I. condensate*, citric acid exudation was significantly correlated with Cu dose, reaching citric acid concentrations 3- to 4-times higher than *L. albus* at 2.0 mg Cu L^{-1}. Meier et al. (2012) attributed the citric acid release in the metallophyte to be a species-specific Cu-tolerance mechanism aimed at chelating the metal to prevent its uptake into the roots. *Oenothera picensis* displayed the highest shoot Cu

concentrations at 116 mg kg^{-1} dry weight (as opposed to 20 mg Cu kg^{-1} dry weight for *L. albus*) and the lowest citric acid concentrations. Instead, *O. picensis* was found to release succinic acid, which has a much lower affinity for Cu; indeed, the stability constant (K_f) of the Cu-succinate chelate is 1100 times lower than that of the Cu-citrate chelate (Meier et al., 2012). From these results, Meier et al. (2012) hypothesized that Cu tolerance could be inferred by looking at the type and quantity of LMWOA released.

2.6.2 Zinc

Among the Zn hyperaccumulators, *Arabidopsis halleri* and *Thlaspi caerulescens* are the most widely studied and have become the model species for understanding metal hyperaccumulation. Under ideal conditions, these plants efficiently extract Zn from both the labile and non-labile soil Zn pools and transport it into their aboveground biomass. Indeed, the shoots of *A. halleri* and *T. caerulescens* can store up to 32,000 or 26,000 mg Zn kg^{-1} dry tissue, respectively (Brown et al., 1995; Zhao et al., 2000). Traditionally, root exudates including LMWOA were believed to increase the bioavailability of heavy metals in the soil through chelation, thus facilitating metal uptake by the hyperaccumulators. However, studies analyzing the root exudates and rhizosphere soil of *A. halleri, T. caerulescens*, and other accumulators have consistently reported no unique changes in the rhizosphere soil compared to the segregated bulk soil in pH, enhanced metal mobility, novel root compounds such as phytochelatins, or increased root exudate secretion (Zhao et al., 2001; Puschenreiter et al., 2003; Dessureault-Rompre et al., 2008; Quartacci et al., 2009). Conversely, metal tolerant and metal excluder species have been shown to increase metal dissolution, decrease rhizosphere pH, and increase organic acid exudation. For example, Quartacci et al. (2009) found that the cultivation of metal contaminated soil with metallicolous species significantly decreased the pH roughly 2 units relative to unplanted controls, while also increasing the extractable Cu and Zn about 3-fold, and Cd 11-fold. Phenolic compounds and flavonoids were also found to be exuded by the metal excluding species, possibly for the acquisition of poorly soluble mineral nutrients such as Fe and P. Conversely, the growth of the metal hyperaccumulator *Brassica carinata* had no effect on soil pH or extractable metal concentrations.

Similarly, Zhao et al. (2001) studied the mobilization efficiency of the root exudates for two ecotypes of the metal hyperaccumulator *T. caerulescens,* and a non-accumulating

wheat species, *Triticum aestivum*. The study found that the root exudates extracted from the roots of the hyperaccumulators mobilized very little Cu, Zn, and Cd from metal contaminated soil, but that the root exudates collected from the non-accumulator removed significantly more Cu, Zn, and Cd, from the soil. Based on root exudate studies of wheat by Marschner (1995) and Ma and Nomoto (1996) they concluded that the exudation of mugineic acid, a phytosiderophore capable of chelating Fe, Zn, Cu and Mn, was responsible for the enhanced metal availability seen with the monocotyledonous plant.

As for the role of root exudates in metal uptake, enhanced metal mobilization in the rhizosphere does not appear to be directly linked to specific mechanisms of hyperaccumulation. Instead, genetically controlled physiological attributes of hyperaccumulating plant species are more important for metal uptake, whereas root exudates likely facilitate metal exclusion through chelation. For example, in one of the first studies analyzing the mechanisms of Zn hyperaccumulation in *T. caerulescens*, Lasat et al. (2000) hypothesized that Zn uptake was linked with specific root membrane proteins. This was based on the observation that plant Zn uptake for barley (*Hordeum vulgare*) and maize (*Zea mays*) followed Michaelis-Menten kinetics, where a maximum rate of uptake was reached indicating saturated conditions. To isolate this Zn transporter, Lasat et al. (2000) used functional complementation between *T. caerulescens* cDNA and a yeast mutant defective in Zn uptake grown in Zn-limiting media. From this study, the gene designated ZNT1 was cloned that could restore growth in low Zn media to the yeast cells upon insertion. Subsequent sequence analysis of the ZNT1 cDNA confirmed it is a member of the micronutrient transporter gene family sharing significant homology with the Fe transporter IRT1, confirming the hypothesis of protein mediated Zn uptake in the root cells of *Thlaspi* species.

In terms of Zn uptake between the *Thlaspi* species, Lasat et al. (2000) later found that the initial Zn influx into the roots of the hyperaccumulator *T. caerulescens* exceeded the non-accumulator *T. arvense* despite the ability of both *Thlaspi* species to transport Zn across the plasma membrane into the root cells. This greater Zn influx for *T. caerulescens* was attributed to a higher concentration of Zn transporters at the plasma membrane of the root cells via greater expression of ZNT1. However, when studying Zn transport over a 48 hour period using the radiotracer ^{65}Zn, Lasat et al. (2000) found that *T. arvense* contained 6-times

the amount of Zn in the roots compared to the hyperaccumulator, while *T. caerulescens* had accumulated 10-fold more Zn in the shoots due to translocation. Upon further study, expression of ZNT1 was also found in the leaf cell plasma of the hyperaccumulator facilitating transport into the shoot cells. These differences in root and shoot Zn concentrations between *T. arvense* and *T. caerulescens* are believed to be a result of complex differences in the Zn transport systems of the two plants, as *T. caerulescens* contained 5-times the amount of Zn in the xylem moving to the shoots. Finally, Lasat et al. (2000) found a high expression of ZNT1 in both the root and shoot cells of the hyperaccumulator *T. caerulescens,* promoting Zn transfer into the roots and within the shoots regardless of Zn status; whereas expression of ZNT1 in *T. arvense* was only stimulated by Zn deficiency.

2.6.3 Cadmium

Unlike Zn and Cu, which are plant micronutrients regulated by metal homeostasis, Cd does not have a physiological function within plants and is toxic at very small concentrations (Prasad, 2004). Nonetheless, both Cd exclusion and hyperaccumulation have been documented for a few plant species (Lasat et al., 2000; Pence et al., 2000; Yang et al., 2004; Basic et al., 2006; Chiang et al., 2006; Zhu et al., 2011; Huguet et al., 2012). Whereas plants do not possess a specific mechanism for Cd uptake, Cd mimics the behavior of other divalent cations in the soil (e.g., Ca^{2+}, Mg^{2+}, Fe^{2+}, and Zn^{2+}), which is why in Cd-tolerant species of *T. caerulescens, A. halleri,* and *Sedum alfredii* Cd hyperaccumulation is linked with Zn hyperaccumulation (Lasat et al., 2000; Pence et al., 2000; Yang et al., 2004; Basic et al., 2006; Huguet et al., 2012). While studying Zn hyperaccumulation in *T. caerulescens,* Lasat et al. (2000) noticed the Zn transporter expressed by ZNT1 also mediated low affinity Cd transport into the root cells. Later, Lombi et al. (2001) found that excess divalent cations reduced Cd uptake for *T. caerulescens*, confirming Cd transport via channels for other ions.

Zhu et al. (2011) used long-term hydroponic and soil experiments to study Cu exclusion by *Lycopersicon esulentum* (tomato). At Cd concentrations between 25 and 50 μ*M*, the Cd-resistant cultivar "Micro-Tom" excreted more oxalate than the Cd-sensitive species "Hezuo903". Moreover, in the Cd-resistant cultivar, oxalate excretion increased with increasing Cd concentration in solution. Conversely, increasing the Cd concentration in solution had no significant effect on oxalate excretions by the Cd-sensitive cultivar. Moreover to examine both the excretion of oxalate and its role in detoxifying Cd, Zhu et al.

(2011) used the anion channel inhibitor PG (phenylglyoxal) to block oxalate release. With the addition of PG to the solution, the "Micro-Tom" cultivar suffered Cd toxicity at 10 μ*M*, as evident by a 30–50% decrease in root elongation and a 44% increase in Cd concentration in the root. The work of Zhu et al., (2011) confirmed the role of oxalate in alleviating Cd toxicity in *L. esulentum*—similar to the role of malate in promoting Al tolerance in Buckwheat (Sasaki et al., 2004).

2.6.4 Aluminum

While aluminum (Al) is not the primary element of concern in smelter-impacted areas, Al toxicity is a major concern in acidic soils where the pH is below 5.5 and the phytotoxic aluminum species Al^{3+} is present (Wang et al. 2006). Aluminum is the third most abundant element in the soil, after oxygen and silicon, and acidification of the soil from sulfuric acid deposition such as in the Creighton/Flin Flon area can solubilize the Al present in aluminosilicate minerals and as oxides and hydroxides, releasing it into the soil solution (Lindsay, 1979; Ma and Furukawa, 2003). In the case of Al tolerance and resistance in higher plants, much of the work to date has focused on agricultural species (e.g., wheat, maize, sorghum, buckwheat, etc.), as acidic soils limit plant production on a significant portion of the world's arable land. Again, contrary to the belief that the organic acid anions are strictly root diffusates and not likely a triggered response to external, environmental stress; studies on Al tolerance and resistance have proven that malate, citrate, and oxalate are specifically involved in Al detoxification where the organic anions complex with Al^{3+} and prevent entry into the root apex or detoxify Al within the plant tissues. The specific response, however, varies with the plant species, organic acid anion species, secretion patterns, and dose response (Ma and Furukawa, 2003). For buckwheat, Zheng et al. (1998) found that Al exposure to seedlings grown in nutrient solution induced oxalate exudation from the root apex of the plants, where phosphorus deficiency and lanthanum (La^{3+}) exposure did not, indicating an Al-specific response. Moreover, root exudates from resistant species of maize exposed to Al in sterile nutrient agar increased with increasing Al concentrations, and consisted of 67% citrate; 29% malate; and 3% trans-aconitate (Mariano and Keltjens, 2003) again indicating Al-specific exudation of the organic acids.

Moreover, the release of malic acid (malate) from the roots of Al-tolerant plants has been has been proven as a mechanisms of Al exclusion from the roots (Sasaki et al., 2004;

Hoekenga et al., 2006; Ligaba et al., 2006). Until recently, the presence of Al-gated anion channels controlling the release of organic acids from the plasma membrane of root apex cells could only be inferred, but not proven. However, Sasaki et al. (2004) used subtractive hybridization between Al-resistant and Al-sensitive wheat varieties to isolate and clone the aluminum activated malate transporter gene (ALMT1). This gene encodes the membrane protein present in the root apex of Al-resistant wheat that facilitates the efflux of malate into the rhizosphere. They then used transgenic cultured tobacco cells expressing the ALMT1 gene to confer Al tolerance to the plant cells, as they exhibited Al-activated malate release, and accumulated less Al than the control cells. Homologues of the ALMT1 gene have also been found in *Arabidopsis*-AtALMT1 and *Brassica napus*—BnALMT1 and BnALMT2, all of which encode aluminum-activated malate transporters on the root cell plasma membrane as a mechanism of Al resistance (Hoekenga et al., 2006; Ligaba et al., 2006).

Adding to the variability between plant species and Al resistance, Magalhaes et al. (2007) used positional cloning to identify a gene from the "multidrug and toxic compound extrusion" (MATE) family in sorghum that confers Al resistance via aluminum-activated citrate transporters expressed in the root apex of the plants. However, the Al-specificity of the SbMATE gene is in question, as MATE proteins have also been involved with the transportation of alkaloids and flavonoids, and could be polyspecific transporters (Magalhaes, 2010). In fact, MATE proteins may also transport other substances—besides citrate—that contribute to Al resistance, such as phenolic compounds (Magalhaes, 2010).

2.6.5 Lead

Like Cd, Pb is a non-essential element for plant nutrition expressing toxicity through the inhibition of root growth by restricting cell division at the root tip. Naturally occurring Pb tolerant plants are typically found on calamine soils, which are strongly enriched in Zn Cd, Pb, and Ca (Reeves, 2006; Mohtadi et al., 2012). Accumulation of Pb in plant tissues, however, is not synonymous with Pb tolerance, and is likely due to the co-occurrence of Ca in calamine soils (Mohtadi et al., 2012). Indeed, exchangeable Ca counteracts Pb toxicity in calamine soils by inhibiting root uptake of the already immobile Pb, thus making Pb accumulation difficult to study (Mohtadi et al., 2012). Mohtadi et al. (2012) also noted that Pb tolerance has not been observed in many metallophytes grown in extremely Pb-enriched environments, which is contrary to the evolution of tolerance for other heavy metals. They

also reported that, as with Cu, most of the documented cases of Pb hyperaccumulation cannot be reproduced under controlled conditions and are likely a result of air-borne contamination. In terms of hyperaccumulation, Mohtadi et al. (2012) did study Pb-tolerant and Pb-sensitive species of *Silene vulgaris*, *Noccaea caerulescens*, and *Matthiola flavida* grown hydroponically. Only one of the Pb-tolerant ecotypes of *N. caerulescens* displayed hyperaccumulation at non-toxic and toxic concentrations of Pb (i.e., 1 to 5 μ*M*), indicating again that hypertolerance and hyperaccumulation are under independent genetic controls (Mohtadi et al. 2012).

In a hydroponic study to assess Pb tolerance in rice, Yang et al. (2000) screened 229 varieties of rice to select three tolerant and three sensitive species. At a Pb concentration of 20 μ*M*, the tolerant species produced 10 times the root biomass compared to the sensitive species by developing adventitious roots after the initial exposure. The presence of these unusual roots lead to the hypothesis that the Pb tolerant varieties had altered the availability of the Pb in solution by altering its speciation, thus allowing the new tissue to grow without Pb toxicity. Yang et al. (2000) subsequently analyzed the hydroponic solutions for LMWOA using HPLC, and found an increase in oxalic acid with increasing Pb concentrations for the tolerant varieties. As a result, they concluded that the tolerant rice varieties were capable of up-regulating the production and secretion of oxalate to inhibit the uptake of Pb into the roots.

2.7 Chemistry at the Soil Root Interface

Previous research into root exudates and root processes, particularly with respect to heavy metals in soil, is limited. Moreover, traditional methods of rhizosphere research use solution cultures to facilitate the collection of root exudates, yet significant differences between solutions and soil limit the applicability of these studies to real world results. For example; soils contain a higher surface area for processes such as adsorption, there are differences in the microbial ecology between soils and solutions, the status of water and oxygen are significantly different between the two systems, and friction caused by soils in the rooting environment can stimulate rhizodeposition, which is absent in solution cultures (Uren, 2007). Thus, with respect to heavy metal contaminants and metalliferous flora, there

is a clear need to characterize the chemistry occurring at the soil–root interface, especially in smelter-affected soils such as in Flin Flon /Creighton.

3 MATERIALS AND METHODS

3.1 Site Selection

The Hudbay Minerals Inc. site lies on the edge of the Boreal Shield Ecozone, at the border of Saskatchewan and Manitoba (Fig. 3.1). This region is characterized by rolling uplands and lowlands on Precambrian granitic bedrock outcrops, moraines, glaciofluvial deposits and colluvial deposits (Zoladeski, 1995). The region has warm, short summers and long, cold winters, which support the growth of white spruce (*Picea glauca*), black spruce (*Picea mariana*), jack pine (*Pinus banksiana*), and tamarack larch (*Larix laricina*). Deciduous trees in the area include white birch (*Betula papyrifera*), trembling aspen (*Populus tremuloides*), and balsam poplar (*Populus balsamifera*). The Flin Flon–Creighton *Green Project* liming sites (Fig. 3.2) are located on accessible rock outcrops with shallow soils, or on glaciofluvial plains. Soils on the rock outcrops and fluvial plains are classified as either Orthic/Cumulic Regosols or Brunisols, while the depressions contain Organic soils (Mycock, 2011). Compared to the undisturbed boreal forest, the smelter-impacted area is heavily eroded, and most of the rock outcrops are exposed with little to no vegetation. The concentrations of heavy metals (Cu, Zn, As, Cd, Hg, Pb, and Ni) vary considerably over the site, especially within a 5-km radius of the smelter. Metal concentrations ranged from 23 to 12,769 mg kg^{-1} for Cu; 231 to 15,716 mg $kg^{-1}$for Zn; 5 to 1,753 mg $kg^{-1}$for As; under detection to 113 mg $kg^{-1}$for Cd; under detection to 44 mg $kg^{-1}$for Hg; 15 to 1,846 mg $kg^{-1}$for Pb; and 8 to 67 mg kg^{-1} for Ni (Bentz and Farrell, unpublished data, 2010). Overall, heavy metal concentrations were highest in concave areas of the landscape, intermediate and wide ranging in poorly developed mineral soils on upper and mid-slope positions, and lowest in developed mineral soils containing a B horizon on upper and mid slopes (Bentz and Farrell, unpublished data, 2010). The sites chosen for this project (Fig. 3.2) represent responsive and non-responsive soils that received a dolostone application in 2000 (Appendix A) (Winterhalder, 2001) and were located about 1- to 2-km from the smelter. In addition, an

undisturbed forest soil (i.e., the "reference" soil) was collected from an area unaffected by the smelter.

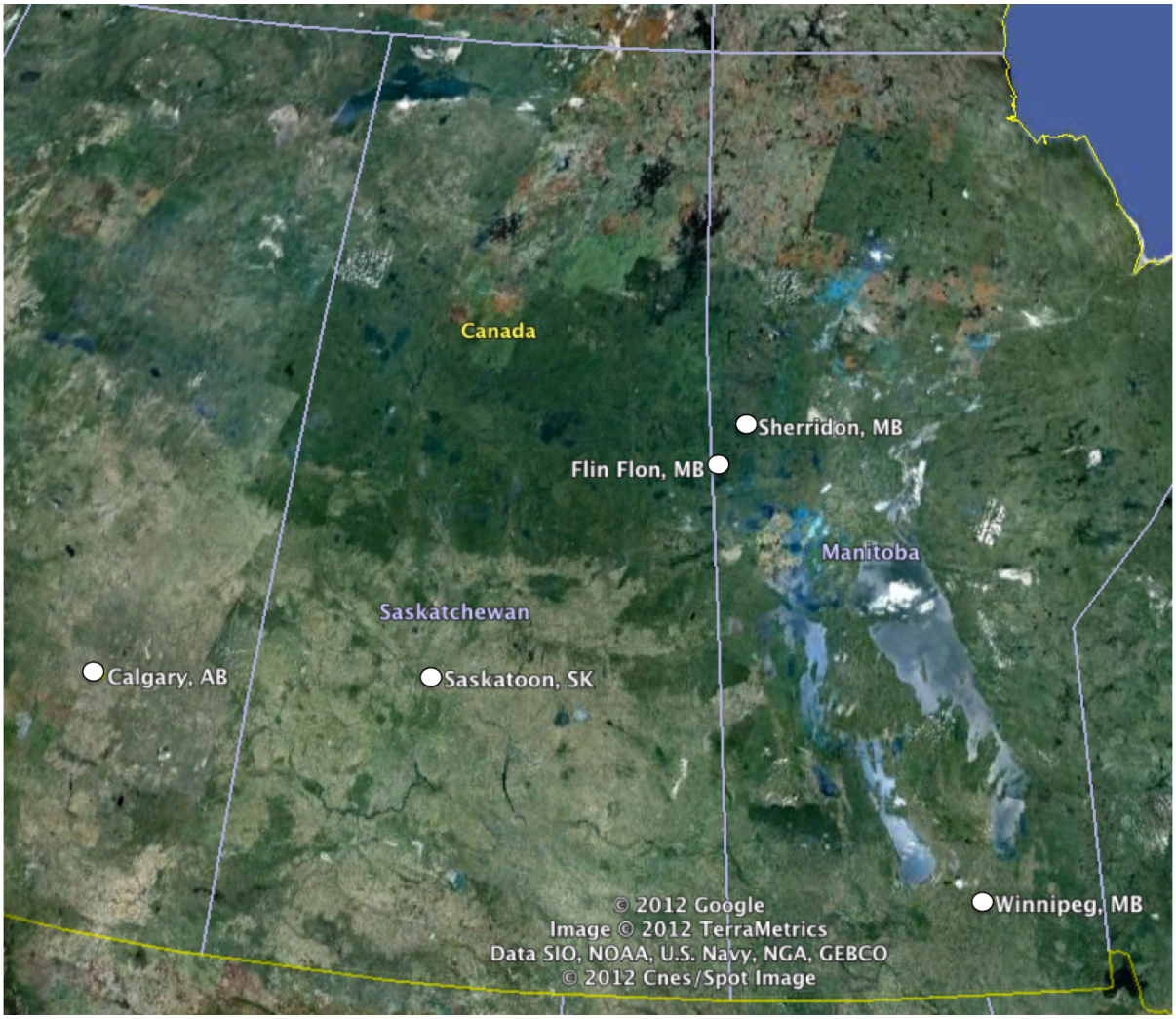

Fig. 3.1. Location of Flin Flon, MB/ Creighton, SK in Canada. Satellite image taken from *Google Earth* (*ver. 6.1.0.5001*, Image acquired 11 October 2011; accessed 03 December 2012).

3.1.1 Reference site (Sherridon)

The reference soil was collected from an area about 40 km northeast of the smelter, near Sherridon, Manitoba. The site is located in a jack pine/lichen ecosite displaying no signs of toxicity or stress (Zoladeski, 1995). The vegetation and soils present are representative of the undisturbed Flin Flon/Creighton area in pre-smelter times with bearberry (*Arctostaphylos*), blueberry (*Vaccinium* section *Cyanococcus*), low bush cranberry

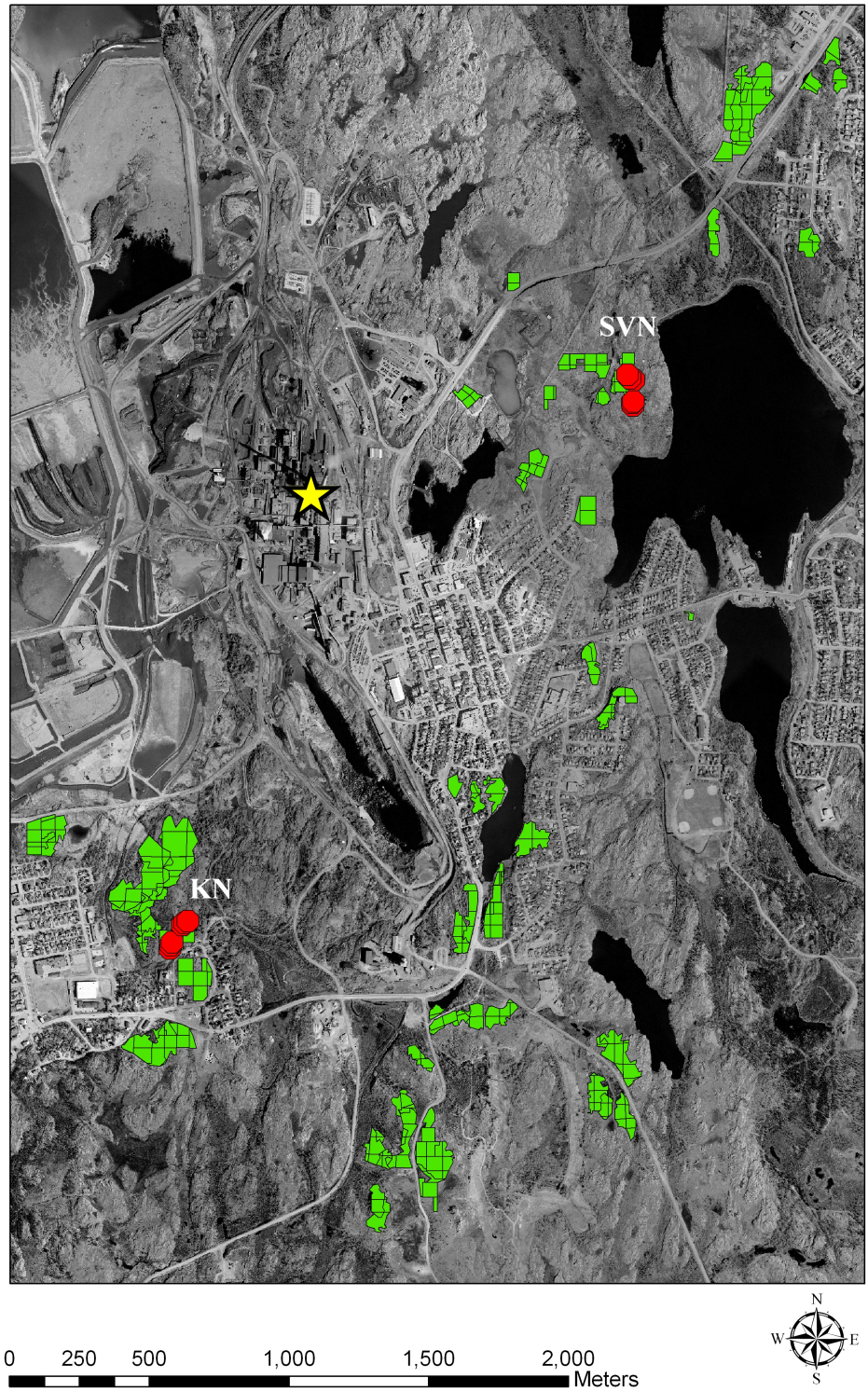

Fig. 3.2. Location of the smelter stack (yellow star), *Green Project* liming sites (green polygons) and 2010 sampling sites (red circles). Second Valley North (SVN) is the lime non-responsive site while Knight North (KN) is the lime responsive site. Aerial photo credit: Hudson Bay Mining and Smelting Inc., 2006.

(*Vaccinium oxycoccos*), sphagnum moss (*Sphagnum*), and small white birch (*Betula papyrifera*) and trembling aspen (*Populus tremuloides*) in the understory. The soil at this site is classified as an Eluviated Dystric Brunisol.

3.1.2 Non-responsive site (Second Valley North)

The Second Valley North (SVN) site is located approximately 1.1 km northeast of the smelter (Fig. 3.2) and the vegetative cover has been very slow to respond to the lime applied in 2000. Vegetation at the site (including both the non-limed and adjacent limed areas) consists of mostly bentgrass and a few seedlings of birch and aspen, but no understory species. The soils at the non-limed and limed sites were classified as Eluvited Dystric Brunisols.

3.1.3 Responsive site (Knight North)

The Knight North site (KN) is located approximately 1.7 km southwest of the smelter (Fig. 3.2). The non-limed area is completely devoid of vegetation—including bentgrass. Soil at the non-limed site was classified as an Orthic Dystric Brunisol, depositional phase, due to the presence of a 10-cm horizon at the soil surface of a different texture and color than the subsoil. This horizon was likely deposited from erosion of the surrounding rock outcrops left barren from forest fires and vegetative dieback. The limed area on the other hand has been very responsive to the dolostone added in 2000, with birch and aspen becoming established only after the dolostone application and reaching 2- to 3-m in height in a period of eight to nine years. Soils on the limed site were classified as Orthic Dystric Brunisols.

3.2 Soil Sampling and Initial Characterization

Soil pits (n = 3) were dug in both the limed and non-limed areas at the SVN and KN sites, and in the undisturbed forest site at Sherridon, in order to classify the soils. Soil from each site was collected for use in the greenhouse trials by first removing any living plants or leaf litter from the surface and sampling the mineral soil (0–15 cm) immediately beneath the plant cover. Soil was collected from a minimum of three locations within each site and then combined to make a composite sample. The soil was air-dried and sieved to ≤ 5 mm for the greenhouse study (to include coarse fragments and carbonates) and ≤ 2 mm for soil characterization. The soils were sieved using Nitex nylon mesh screens (Dynamic Aqua-Supply Ltd., Surrey, BC). The coarse fragments and carbonates were included in the growth

chamber study to maintain the physical characteristics of the rooting environment found in the field. Soils were collected in July 2010.

3.2.1 Particle size analysis

The particle size distribution was measured according to Degenhardt (2010) on the air-dried, sieved (≤ 2 mm) soil. The samples were pretreated to remove organic matter (Kroetsch and Wang, 2008) by treating 0.5 g of air-dried soil with 5 mL of 35% (w/w) hydrogen peroxide (H_2O_2) and heating the samples on a hot plate. The H_2O_2 additions were repeated 3–5 times; i.e., until the samples stopped effervescing. The samples were then heated to dryness at 90°C. Immediately before analysis, 10 mL of double deionized water and 0.5 mL of dispersing agent [i.e., 0.065 *M* sodium hexametaphosphate, $(NaPO_3)_6$] were added to the pretreated soils and the resulting slurries sonicated for 15 s using a Branson Sonifier Analog Cell Disrupter 350 (Branson Ultrasonics Corp., San Francisco, CA). The particle size distribution was then measured via laser diffraction using a LA-950 Laser Scattering Particle Size Distribution Analyzer (Horiba Instruments Inc, Irvine, CA.) following the manufacturer's instructions. Note: because the soils were quite acidic, pretreatment to remove carbonates and soluble salts was not necessary.

3.2.2 Soil pH

Soil pH was analyzed using a PC700 series pH/mV/Conductivity/Temperature benchtop meter and a Gel 3-in-1 pH electrode (Oakton, Thermo Fisher Scientific, USA). A 1:2 (w/w) soil:0.01*M* $CaCl_2$ suspension was prepared as described by Hendershot et al. (2008). The mild $CaCl_2$ solution was chosen over deionized water due to the high content of metal sulfide salts likely present from smelting activities, which can interfere with pH measurements—and for non-saline soils, the pH measured in 0.01*M* $CaCl_2$ is relatively independent of soluble salt concentrations (Hendershot et al., 2008).

3.2.3 Exchangeable cations and available potassium

Cation exchange capacity (CEC) was measured using un-buffered 1.0 *M* NH_4Cl to extract the air-dried soils; the soil:solution ratio was 1:40 (w/v) as described by Skinner et al. (2001), who reported that it was the most accurate method for measuring the CEC of acidic forest soils where pH dependent changes need to be considered. The soil:NH_4Cl suspension was shaken for 24 h at 150 rpm, centrifuged at 900 rpm for 15 min, and then vacuum filtered

through 0.45 μm Millipore filters. The extracts were analyzed for calcium (Ca^{2+}), magnesium (Mg^{2+}), potassium (K^{+}), sodium ($Na^{+)}$, aluminum (Al^{3+}), copper (Cu^{2+}), zinc (Zn^{2+}), cadmium (Cd^{2+}), and lead (Pb^{2+}) using atomic adsorption spectroscopy (Spectra AA220 Atomic Absorption Spectrometer; Varian Australia Pty Ltd). The total CEC ($cmol_{(c)}$ kg^{-1}) was then measured by extracting the soil-retained ammonium (NH_4^+) with 2 *M* KCl, after washing the soil three times with 50 mL of double deionized water to remove any residual NH_4Cl. The NH_4^+ in the KCl extracts was analyzed colormetrically by reaction with alkaline phenol and hypochlorite using the SmartChem 200 auto-analyzer (Westco Scientific Instruments Inc., Brookfield, CT). Base saturation was calculated as the percent of the CEC occupied by Ca^{2+}, Mg^{2+}, K^+, and Na^+.

3.2.4 Available nitrogen

Available N was determined using air-dried soils sieved to ≤ 2 mm. Inorganic N was extracted using a 2 *M* KCl extracting solution at a 1:10 (w/v) soil:solution ratio (Maynard et al., 2008). The samples were shaken for 30 min at 160 rpm and filtered through Whatman No. 42 filter paper. The NH_4^+ and NO_3^- contents of the extracts were determined using the SmartChem 200 auto-analyzer.

3.2.5 Total sulfur

Total sulfur was determined using air-dried soil that was sieved to ≤ 2 mm and then finely ground with an agate mortar and pestle. The soils were mixed with a catalyst (Com-Cat 502-321) in a 1:4 (w/w) soil:catalyst ratio and analyzed by combustion at 1450°C using a LECO CNS-2000 Elemental Analyzer (LECO Corporation, St. Joseph, MI).

3.2.6 Total organic carbon and inorganic carbon

Total and organic soil C were determined using a dry combustion technique in which the soil C is thermally oxidized to form CO_2 which is then quantified using infrared gas analysis (Wang and Anderson, 1998; Skjemstad and Baldock, 2008). Organic C can be digested within 120 seconds with a small sample size (0.2-0.4 g) before the carbonates start to break down. Given that the soils in Flin Flon developed on Precambrian bedrock, which is naturally acidic and base poor, it is likely that few carbonates are present in the samples as a consequence of the parent material. However, with the additions of dolostone to the surface of the soils by the *Green Project,* differences in the inorganic C content of the soils is

expected between the limed and non-limed sites. The presence of dolostone in the samples may complicate the C analysis because, unlike calcium carbonate, dolostone partially decomposes at temperatures between 750-830^{o}C causing an overestimation of the organic C content (Wang and Anderson, 1998). This was not case, however, as the dolostone (which was added to the surface of the field soils) was removed during sampling; any inorganic C that had translocated into the soil upon dissolving likely reacted with hydrogen ions in the acidic soil. Organic and inorganic soil carbon can be differentiated based on the temperature used to combust the sample.

Organic carbon content and total carbon were analyzed consecutively on the LECO Cr-12 Carbon Analyzer (LECO Corporation, St. Joseph, MI) via combustion and CO_2 analysis at 842°C and 1142°C respectively.

3.2.7 Extraction and analysis for heavy metals

Total trace element concentrations in the soils were determined using microwave digestion (ETHOS One; Milestone S.r.l. Sorisole Bergamo, Italy) followed by analysis using microwave plasma atomic emission spectroscopy (MP-AES) (Agilent Technologies, Australia).

For sample digestion, 0.5 g of air-dried, ground soil was placed into Teflon vessels containing 12-mL aqua regia (i.e., a 3:1 mixture of concentrated HCl and HNO_3); the vessels were sealed and the samples digested at 200°C under pressure. The digests were filtered through Whatman No. 41 filter paper, diluted to 50 mL with double deionized water, and analyzed using MP-AES as per the manufacturer's instructions. The accuracy of the digestion and analysis was verified using standard reference materials (SRM 2710a and SRM 2781) obtained from the National Institute of Standards and Technology (NIST; Boulder, CO) in 2009.

3.2.8 Plant available heavy metals

Plant available heavy metals (Cu^{2+}, Zn^{2+}, Cd^{2+}, Al^{3+}, Pb^{2+}, and Ni^{+}) in the soil were determined using a desorption-replenishment technique with 0.01*M* $CaCl_2$ as the extracting solution. The choice of 0.01*M* $CaCl_2$ as the extracting solution reflects the facts that (i) it has a constant electrolyte concentration, which mimics the ionic strength of most soil solutions, so that the metals in solution reflect their binding strength and solubility; (ii) Ca^{2+} is one of

the primary cations in the soil and is efficient in replacing (exchanging) other cations on the soil adsorption complexes; and (iii) multi-elemental analysis from a single extraction allows for the interpretation of relationships between metals and their influence on one another (Houba et al., 2000). The $CaCl_2$ extracting solution was unbuffered to ensure that pH-dependent effects on metal availability would be negligible.

Air-dried soil (sieved to ≤ 2 mm) was extracted with 0.01*M* $CaCl_2$ at a 1:10 (w/v) soil:solution ratio using a method adapted from Houba et al. (2000), Strawn and Hansen (2003), and Koo et al. (2010). The samples were shaken at 160 rpm for two hrs, centrifuged at 900 rpm for 15 min, and the clear supernatant vacuum filtered through a 0.45 μm Millipore filter. The soil and solution remaining in the centrifuge tube were then weighed, enough fresh extracting solution was added to bring the soil and remaining solution back to its original weight, and the shaking/extraction procedure repeated for a total of 10 extraction cycles. Each individual extract was analyzed using MP-AES (Agilent 4100 MP-AES, Agilent Technologies, Australia).

Analysis of the initial soils data indicated that despite lower total concentrations (i.e., the percent recovery of total available metals was 84–100 % for Zn, 60–75% for Cu, 70–90 % for Cd, 24–80% for Al, and 40–81% for Ni), the differences between soils were clearly evident after only five extraction cycles. Thus, for logistical reasons—and following the initial soil characterization (i.e., for the growth chamber studies)—the number of extraction cycles was reduced to five—with all five extracts combined to yield a composite sample for analysis.

3.3 Greenhouse Studies

3.3.1 Experimental design

The three metal-tolerant plant species selected (*S. vulgaris, A. capillaris,* and *P.* sp. 'Frensham') were grown in 15-cm pots containing 1.5 kg of soil (sieved to ≤ 5 mm). The experiment was structured as a 2-way randomized complete block design with three main treatments (plant species) and five sub-treatments (soil/dolostone combination) replicated six times. In addition, an unplanted control (n = 6) was included for each soil and was watered regularly with the planted pots. [Note: due to the presence of fine roots throughout the pots of the planted soils, the unplanted soils were used as a "bulk", non-rhizosphere, soil.]

Seeds for the Cu-tolerant Imsbach population of *S. vulgaris* were kindly donated by Dr. Henk Schat (Vrije Universiteit, Amsterdam, The Netherlands); *A. capillaris* seeds were collected from the SVN site (Flin Flon, MB) in August 2011. Both the *S. vulgaris* and *A. capillaris* seeds were germinated in zeolite and transplanted at the two-leaf stage; planting rates were 10 and 5 plants per pot, for *S. vulgaris* and *A. capillaris*, respectively.

Propagated plants of *P* sp. 'Frensham,' a sterile hybrid, were obtained from Richters Herbs (Goodwood, ON, Canada) and transplanted upon arrival after cleaning the roots of potting soil. The plants were grown in the greenhouse and initially watered with a modified, ¼-strength Hoagland's solution [1.5 m*M* KNO_3, 1.0 m*M* $Ca(NO_3)_2$, 0.5 m*M* $MgSO_4$, 0.25 m*M* $(NH_4)H_2PO_4$, 11.5 μ*M* H_3BO_3, 2.3 μ*M* $MnCl_2$, and 0.035 μ*M* $NaMoO_4$; Hoagland and Arnon, 1938] three times per week until established; thereafter—and until harvest—the plants were watered as needed with tap water. [Note: given the high concentrations of the trace metal micronutrients present in the soils, these micronutrients were not added in the plant nutrient solution.]

3.3.2 Plant harvest for roots, shoots, and root-affected soil

Plants were harvested during the vegetative life cycle stage—which varied between 7 and 12 wks, depending on the plant species—soil type, and metal stress during establishment. The vegetative stage was chosen to sample the period of maximum root exudation, which occurs early in the developmental stages and decreases with age as photosynthetic C is converted from nutrient acquisition to reproduction and ripening (Swinnen, 1994; Gransee and Wittenmayer, 2000; Aulakh et al., 2001; Nguyen, 2003; Kerdchoechuen, 2005). At harvest, the shoots were removed and carefully washed with deionized water before drying. Collection of the roots and root-affected soil proved to be more difficult. Typically, root-affected (rhizosphere) soil is defined as the volume of soil that adheres to the roots after extraction and gentle shaking (Szmigielska et al., 1996; Kloepper and Beauchamp, 1992; Gillespie, 2003). This extraction technique worked well for plants with large, fibrous root systems like *A. capillaris*, but did not work well for smaller plants with tap root systems, like as *S. vulgaris* and *P.* sp. 'Frensham'. A preliminary growth study was conducted with the lime-responsive soil from the KN area to optimize growth conditions and test sampling protocols for the collection of shoots, roots, and root-affected soil for all three plants. For both *S. vulgaris* and *P.* sp. 'Frensham', very little "rhizosphere" soil could be collected from

the tap root, while the secondary roots tended to break off and were dispersed throughout the soil. To maximize the collection of possible root-affected soil, all the soil in the pots was considered "root-affected" for organic acid extraction and concentration, while the bulk soil was collected from the un-planted pots.

At harvest, the soil in each pot was divided into two equal sub-samples; the roots and small stones in each sub-sample were then removed by sieving the soil through a 2-mm nylon mesh screen and the soil placed into 1-L polypropylene bottles containing 700-mL double deionized water. Soil adhering to the roots was transferred into the 1-L polypropylene bottles by placing the nylon mesh in the water and gentling shaking. The roots were then bulked for each pot, washed with deionized water to remove any remaining soil, and dried.

3.3.3 Collection, identification, and quantification of low molecular weight organic acids using anion exchange membranes

Low molecular weight organic acids were isolated by solid phase extraction (SPE) using anion exchange membranes (AEM) and quantified via derivatization and GC-FID analysis (Szmigielska et al., 1996, 1997; Gillespie, 2003). Anion exchange membranes obtained from Western Ag Innovations (Saskatoon, SK, Canada) were cleaned by shaking in 0.5 *M* HCl for 8 h, decanting off and replacing the HCl with fresh 0.5 *M* HCl and repeating the process for a total of three wash cycles. The AEM were then regenerated by soaking the membranes in 0.5 *M* NaOH and shaking for 8 h, decanting off and replacing the NaOH with fresh 0.5 *M* NaOH and repeating the process for a total of three wash cycles.

A single AEM was placed into each of the polypropylene bottles containing the soil/water slurries (see Section 3.3.2) and shaken on a rotary shaker for 12 h. The membranes were removed, rinsed with deionized water, and eluted with 5 mL of 0.5 *M* HCl with the eluate collected in 6-mL glass vials and stored at 4°C until analyzed. The soil/water slurries were transferred to 23 × 33 × 5 cm aluminum pans and air-dried. The dried soil from the two halves of each pot were then weighed, bulked together, and homogenously mixed for pH measurements, and total- and available- metal extractions.

3.3.4 Organic acid extraction and derivatization

Organic acids were extracted from the AEM eluate, derivatized with *N*-methyl-*N*-[*tert*-butyldimethylsilyl]-*N*-methyl-trifluoroacetamide (M-TBSTFA), and analyzed via GC-FID according to the procedure developed by Gillespie (2003). Derivatization of the organic acids

is required, as the LMWOA are not intrinsically volatile and therefore not amenable to GC-FID analysis. Derivatization with M-TBSTFA replaces the active hydrogen atoms on the polar functional groups (e.g., -COOH, -OH) with the nonpolar molecule $Si(CH_3)_2C(CH_3)_3$ (Blau and Halket, 1993). The LMWOA were extracted by transferring a 1-mL aliquot of the acidified membrane eluate into a 4-mL vial containing 100 µL of 100 ppm glutaric acid (used as an internal standard), adding 2 mL of diethyl ether (DEE) and shaking for 10 s to extract the organic acids from the aqueous phase. The aqueous and non-aqueous phases were allowed to separate before the non-aqueous phase was transferred into a 2-mL autosampler vial containing 40 µL of triethylamine (TEA) and evaporated under N_2 at room temperature. The membrane eluate was then extracted twice more to achieve quantitative (i.e., 99.9%) recovery. The LMWOA were then derivatized by dissolving the DEE residue in 75 µL of isooctane before adding 25 µL of M-TBSTFA. The vial was capped immediately and heated at 70°C for 45 min for derivatization. The vials were allowed to cool before the *t*-BDMS ester derivatives were analyzed using GC-FID.

Standard curves were generated using standard solutions of each organic acid at concentrations of 0.01, 0.05, 0.08, 0.10, 0.30, 0.50, 0.80, 1.0, and 5.0 ppm (Appendix B). For each standard, a single activated AEM was placed in 1.0 L of standard solution and equilibrated by shaking for 12 h. The membrane was then removed, rinsed with double deionized water, and eluted with 5 mL of 0.5 *M* HCl. The membrane eluates were extracted and derivatized as described above, and then analyzed using GC-FID as described in Section 3.3.5. The limit of detection for each organic acid was defined as the lowest concentration of analyte with the AEM producing a peak height twice the amplitude of the base noise in the blank isooctane run (McNair and Miller, 1998). The limits of detection (*LOD*) were calculated from the noise (N), sensitivity (S), and peak width at half height (W_h) for each acid according to Colón and Baird (Table 3.1) (2004):

$$LOD = \frac{3N}{SW_h}$$

3.3.5 GC-FID analysis of *t*-BDMS ester derivatives

Samples were analyzed on a Varian CP-3800 GC-FID equipped with an Varian 8400 autosampler. A Varian FactorFour™ column (VF-Xms) was used for analytical separation with dimensions of 30 m × 0.25-mm i.d. and a 0.25-µm stationary-phase film thickness. Ultra

high purity helium was used as the carrier gas (at a constant flow rate of 2 mL min^{-1}) and the make-up gas (at a flow rate at 25 mL min^{-1}). The injection port temperature was set at 300°C. A 1 µL splitless injection volume was delivered with a 10 µL syringe. The initial column temperature was held at 60°C for 2 min, then ramped at 4°C min^{-1} to 300°C and held for 5 min, for a total run time of 67 min. Low molecular weight organic acids of interest are oxalic, malonic, maleic, succinic, fumaric, malic, tartaric, *trans*-aconitic, and citric. Analysis of all the unplanted controls found no detectable LMWOA.

Table 3.1. Limits of detection and retention times for *t*-BDMS ester derivatives of low molecular weight organic acids recovered with anion exchange membranes.

Organic Acid	Detection Limit	Retention Time
	— ng s^{-1} —	— min —
Oxalic	1.0906	20.89
Malonic	0.0355	23.39
Maleic	0.0262	25.99
Succinic	0.0236	26.27
Fumaric	0.0120	29.09
Malic	0.1466	33.84
Tartaric	0.2157	39.43
Trans-Aconitic	0.0178	39.58
Citric	0.1343	42.27

3.3.6 Root and shoot analysis for total metals

Dried roots and shoots were ground in liquid N using an agate mortar and pestle, then digested in sulfuric acid in 75 mL digestion tubes at 360°C according to the procedure developed by Thomas et al. (1967). For digestion, 0.300 g of plant material was digested in 5 mL of concentrated sulfuric acid (18*M* H_2SO_4) at 360°C for 30 min. Once the plant matter had broken down, 2 mL additions of hydrogen peroxide (30 % v/v H_2O_2) were added and the samples heated for 30 min, or until the digests were clear—indicating that all the carbon had been oxidized. The digests were heated for another hour to remove all traces of H_2O_2, cooled, and brought to volume with double deionized water. The samples were vacuum filtered through 0.45 µm Millipore filters and analyzed for Cu, Zn, Cd, Al, and Ni using MP-AES (Agilent 4100 MP-AES, Agilent Technologies, Australia).

3.3.7 Soil analysis

The dried root-affected soils from the soil/water slurries were analyzed for pH, total extractable metals, and for plant available metals after grinding and homogenization with an agate mortar and pestle. The procedures used are described in Sections 3.2.1, 3.2.7 and 3.2.8, respectively.

3.4 Statistical Analysis

All statistical analyses were performed using IBM® SPSS© Statistics (Version 20). All data were checked for normality using the Shapiro-Wilk test ($P \leq 0.05$) and for homogeneity of variance using Levene's test ($P \leq 0.05$). The conditions of normality and homogeneity of variance were not met for most of the populations—including soil pH, root and shoot weights, root and shoot metal concentrations, total extractable metals and available metals from the soil, and the concentrations of LMWOA in the soil. Given the differences in physical and chemical characteristics between the five soils as well as the different mechanisms and tolerances to heavy metals between the plants, this high degree of variability in the data was not unexpected. Log transformations of the measurements did not improve normality or homogeneity of variance. Therefore, non-parametric statistics were used to determine differences in the distribution of the ranked data between the reference soil and the non-limed soils, the non-limed soils and their limed counterparts, and the reference soil and limed soils to satisfy Objective 1. To satisfy Objectives 2a and 2b, differences between the three plants on each of the five soils were analyzed. The Kruskal-Wallis test ($P \leq 0.10$) was used to analyze differences in the distribution of the data while the Games-Howell test ($P \leq 0.10$) was used to determine which populations were statistically different. Spearman's rank correlation (r_s) was used to determine the strength of the relationships between the variables measured. For the LMWOA, extreme outliers for individual organic acids were found randomly throughout the data. These were assumed to be contamination from the AEM membranes that were used to establish the calibration curves (Appendix B) and, hence, were removed prior to data analysis; extreme outliers accounted for only 2.5% of the data. For the plant samples, there were six replicates of each plant except for *S. vulgaris* on the SVN non-limed soil where the shoots from one replicate were lost during harvest (n = 5).

4 RESULTS AND DISCUSSION

4.1 Soil Characteristics

Data from the initial soil characterization are summarized in Table 4.1; images of the vegetation and soils at each site are presented in Appendix A. Soil at the reference site was classified as an Eluviated Dystric Brunisol and the stand was dominated by jack pine (*Pinus banksiana*), with bearberry (*Arctostaphylos*), and reindeer lichen (*Cladonia rangiferina*) in the understory. The vegetation at this site represents the target for the *Green Project*; i.e., a return of the smelter-affected area to a healthy boreal forest. The pH, nutrient status, base cations, and heavy metal content in this soil are low, which is typical for sandy, mineral soils under jack pine (Schmidt et al., 1996; Borge, 1997; Nesdoly and Van Rees, 1997). Previous studies assessing nutrient regimes for Brunisols (surface 0–20 cm) report pH values between 3.7 and 5.2; NH_4^+ concentrations of 2.1–21.3 mg kg^{-1}, NO_3^- concentrations of 0.3–1.0 mg kg^{-1}; exchangeable Ca^{2+}, Mg^{2+}, and K^+ concentrations of 0.04–6.4, 0.02–1.2, and 0.06–0.8 $cmol_c$ kg^{-1}, respectively, which resulted in base saturations of 35.1–57.6% (Schmidt et al., 1996; Borge, 1997; Nesdoly and Van Rees, 1997). Base saturation in the reference (Sherridon) soil was 2- to 2.5-times greater than that in the non-limed soils from the SVN and KN sites, respectively. As expected, liming the soils significantly increased base saturation in the SVN and KN soils—resulting in %BS values that were 1.3- to 2.5-times greater than that of the reference soil.

Total metal concentrations in the smelter-impacted soils at SVN and KN were generally high (Table 4.1), and were much greater than the concentrations at Sherridon—this was especially true of Cu, Zn, and Pb. Available metal concentrations were generally very low in the reference (Sherridon) soil, and greatest in the lime non-responsive SVN soil. Moreover, in the limed soils, available metals accounted for a much smaller proportion of the total metals than they did in the non-limed soils (Table 4.1). Relatively high concentrations of Cu and Zn in the reference soil presumably reflects (i) natural enrichment of these metals in the area soils and (ii) deposition of smelter-derived metals, which are known to transport up to 100 km from the smelter (Zoltai, 1988).

Table 4.1. Physical and chemical characteristics, and metal concentrations in soils from the Sherridon site (reference), the Second Valley North site (lime non-responsive), and the Night North site (lime responsive) from 0-15 cm depth.

Soil Property (n = 2)†	Sherridon	- Second Valley North -		-- Knight North --	
	Reference	Non-limed	Limed	Non-limed	Limed
pH	4.04	4.01	4.25	3.36	5.10
Sand (%)	64.90	48.86	64.00	22.93	21.15
Silt (%)	32.13	51.15	28.21	66.76	78.15
Clay (%)	0.13	0.00	0.12	10.32	0.72
Texture	SL	SL	SL	SiL	SiL
TOC‡ (%)	0.69	0.86	0.74	3.70	1.01
Total S (%)	0.00	0.02	0.01	0.22	0.03
NO_3^- (mg kg^{-1})	0.00	0.07	0.00	8.85	1.69
NH_4^+ (mg kg^{-1})	20.58	7.25	14.34	8.58	8.45
CECe § ($cmol_c$ kg^{-1})	2.62	4.15	4.45	8.48	8.16
Exchangeable Ca ($cmol_c$ kg^{-1})	0.59	0.52	1.10	0.48	4.06
Exchangeable Mg ($cmol_c$ kg^{-1})	0.28	0.23	1.01	0.60	3.65
Exchangeable K ($cmol_c$ kg^{-1})	0.12	0.10	0.08	0.13	0.10
Exchangeable Na ($cmol_c$ kg^{-1})	0.07	0.05	0.12	0.18	0.35
Base Saturation (%)	40.51	21.90	52.18	16.34	99.88
Total Cu (mg kg^{-1})	11.47	214.2	85.53	451.4	386.1
Available Cu (mg kg^{-1})	0.09	51.68	9.16	102.5	1.65
Total Zn (mg kg^{-1})	35.76	551.2	296.3	792.4	567.1
Available Zn (mg kg^{-1})	1.20	417.4	154.8	56.80	6.05
Total Cd (mg kg^{-1})	0.34	4.99	2.85	12.33	8.03
Available Cd (mg kg^{-1})	0.25	3.22	1.57	1.51	1.11
Total Pb (mg kg^{-1})	0.00	51.00	27.00	836.0	111.0
Available Pb¶ (mg kg^{-1})	0.00	19.6	1.6	8.0	4.0
Total Ni (mg kg^{-1})	0.24	1.02	0.41	0.47	0.42
Available Ni (mg kg^{-1})	0.00	0.00	0.00	0.00	0.00
Total Al (mg kg^{-1})	2898	3037	3282	3748	3462
Available Al (mg kg^{-1})	78.33	79.29	52.23	460.0	8.02

† (n = 2) for all the measurements except for total S and TOC in which (n = 3).

‡ TOC = Total organic carbon

§ CEC_e= effective cation exchange capacity

¶ Available Pb was measured using flame atomic absorption spectroscopy due to detection limitations on the MP-AES.

4.1.1 Non-limed soils

The non-limed soil at SVN and the reference soil from Sherridon were similar in terms of texture and pH; likewise, the two soils had similar concentrations of exchangeable base cations, available Al^{3+}, and TOC (Table 4.1). However, where these soils differed was in nutrient status, base saturation, and heavy metals concentration. The non-limed SVN soil had low available N (NO_3^- + NH_4^+) and a base saturation of only about 22%. The SVN soil was also very high in available metals, with concentrations of Cu, Zn, Cd and Pb that were 572×, 348×, 13×, and 20× greater, respectively, than the reference soil—and which combined with the inherently low soil fertility, likely contributed to the lack of vegetation at the site. The high concentrations of Zn, Cd, and Pb were likely due to the proximity of the site to the former Zn settling pond east of the smelter.

The non-limed soil at the KN site, on the other hand, was substantially different from the reference soil (Table 4.1), with similar concentrations of only the base cations—which are naturally low in forest soils. The KN soil also had the lowest pH (3.36), % base saturation (16.34%), and the highest clay content (10%), TOC content and total S content (0.22%) of the non-limed soils. Concentrations of available Cu, Zn, Cd, Pb and Al were 1140×, 47×, 6×, 8×, and 6× greater, respectively, in the KN soil than the reference soil. The extremely high concentration of available Cu in this soil is likely due to the proximity of the site to the copper slag piles south of the smelter. The high TOC content is due to layers of charcoal found in the soil profile (see Fig. A3; Appendix A).

4.1.2 Limed soils

At SVN, the application of dolostone by the *Green Project* produced a small increase in soil pH; i.e., soil pH increased from 4.01 to 4.25. Moreover, despite the physical addition of Ca and Mg to the soil on the limed site, the exchangeable Ca^{2+} and Mg^{2+} increased by only 0.58 and 0.87 $cmol_c\ kg^{-1}$, respectively. However, given the relatively low CEC of the soil (8.16 $cmol_c\ kg^{-1}$), this resulted in a large increase in base saturation (i.e., from 21.9% to 52.2%). Likewise, concentrations of available Cu, Zn, Cd, Pb and Al in the limed soil were 82%, 63%, 51%, 92%, and 34% lower, respectively, than the non-limed soil. Somewhat surprisingly, total metal concentrations in the limed soil decreased as well, which may reflect displacement of metals on the soil exchange complex and their subsequent migration into the subsoil.

At the KN site, the pH of the soil increased from 3.36 to 5.10 during the ten years following application of the dolostone—giving this soil a pH more than 1-unit higher than that of the Sherridon or SVN soils. Concentrations of exchangeable Ca^{2+} and Mg^{2+} increased as well (by 3.58 and 3.05 $cmol_c\ kg^{-1}$, respectively), with a concomitant increase in base saturation from 16.3% to 99.9%. At the same time, concentrations of available metals in the limed soil decreased drastically (Table 4.1); i.e., concentrations of Cu, Zn, Cd, Pb and Al in the limed soil were 98%, 89%, 26%, 50% and 98% lower, respectively, in the limed soil than in the non-limed soil. Liming the KN site also produced significant decreases in total metal concentrations.

It is difficult to directly compare the response to liming in Flin Flon, MB/Creighton, SK to its parent liming program in Sudbury, ON due to a few key differences between the sites. In Sudbury the smelting of Ni and Cu resulted in high total concentrations of these metals in the soil (up to 29,000 and 3850 mg kg^{-1}, respectively) but not Cd (≤ 2.45 mg kg^{-1}) or Zn (≤ 115 mg kg^{-1}) resulting in different contaminants of concern at these sites (Center for Environmental Monitoring, 2004). Moreover, once the liming had proved effective in alleviating metal toxicity and increasing the pH of the soils at Sudbury the revegetation project commenced with finely crushed limestone, fertilizer, and seeding/tree planting, mitigating the issues of dolostone dissolution, poor nutrient status, and lack of a viable seed source (Winterhalder, 1996). At Sudbury, the effects of lime itself were not studied on soils in the field, just in germination tests in the greenhouse. However, field amendment studies around the industrial complex in Puchuncaví Valley, Chile have studied the effects of lime [$Ca(OH)_2$] and compost in the revegetation of metal enriched soils (Córdova et al., 2011; Ulriksen et al., 2012). After seven months, both studies reported that the incorporation of lime decreased the KNO_3^--extractable Cu^{2+} by several orders of magnitude, but that liming itself resulted in no significant increase in plant biomass of native species (i.e., *Chrysanthemum coronarium*, *Lolium temulentum, Lolium multiflorum*, and *Eschscholzia californica*) compared to the non-amended reference soil. Only the lime + compost treatment produced a significant increase in plant biomass along with a significant decrease in KNO_3^--extractable Cu^{2+}. This was attributed to the addition of organic matter to the soil (Córdova et al., 2011; Ulriksen et al., 2012). Similarly, in a greenhouse study with blue bunch wheatgrass (*Agropyron spicatum*) in soils from the Cu-smelting region of Anaconda, MT, Redente and

Richards (1997) studied the effects of lime and fertilizer on plant growth in pots containing surface (0–5 cm) or subsurface (5–20 cm) soils. Thirteen weeks after $CaCO_3$ was mixed into the soils, the pH in all of the soils had increased; likewise, there was a decrease in the HCO_3^--DTPA extractable Zn in the subsurface soils. However, as was the case with the Chilean soils, applications of lime alone did not increase plant biomass in any of the trials. Only the addition of fertilizer significantly increased plant biomass—demonstrating the importance of nutrient limitations on smelter-affected soils (Redente and Richards, 1997).

The difference between the two sites in Flin Flon in their response to liming is likely a result of the combined effects of soil acidity, initial vegetation, texture of the dolostone, nutrient status, and metal speciation. Dolostone is relatively insoluble in water and requires direct contact with the soil, specifically the hydroxide ion, to dissolve, and is usually mixed into the surface of the soils during application (Thomas and Hargrove, 1984). The more acidic a soil, the faster the dolostone will react and produce a greater increase in pH. For the KN site, the low initial pH (3.36), complete lack of native vegetation, and higher N content facilitated the dissolution of the coarse dolostone, as evidenced by the 1.71 unit increase in pH, high concentration of Ca and Mg in the soil, and the establishment of birch and aspen on the limed soil. Moreover, the soils at this site are located in small depressional catchments on rock outcrops, which restricts leaching. Thus, the observed change in total metal concentrations most likely reflects the uptake and accumulation of these metals by the birch and aspen that became established after liming. Indeed, both birch and aspen have been shown to accumulate metals in their aboveground tissues, effectively removing them from the soil (Kozlov et al., 2000; Rosselli et al., 2003; Evangelou et al., 2012). Birch especially has been shown to extract Zn, Cd, and Pb from metal contaminated soil in phytoextraction/phytostabilization studies (Kozlov et al., 2000; Rosselli et al., 2003; Evangelou et al., 2012), and could account for the change in total metals in the non-limed and limed soils (Table 4.1). Copper on the other hand, is typically highly controlled within plants, and shoot concentrations do not change between normal and contaminated soils for healthy plants, however the micronutrient is still removed from the soil by plants for metabolic purposes. The speciation of the metals may also differ between the sites. Indeed, in a parallel study, Hamilton (unpublished data, 2012) found that Zn speciation at the KN site changed from predominantly franklinite ($ZnFe_2O_4$; 62%) with secondary concentrations of

sphalerite (ZnS; 12%) and Zn-Al-hydroxy interlayer material (Zn-Al-HIM; 26%) in the non-limed soil at a pH of 3.8, to predominantly Zn-Al-HIM (49%), with secondary concentrations of franklinite (25%) and Zn adsorbed to silica oxide bonds (SiO_2; 26%) in the limed soil at a pH of 4.3. Most importantly was the formation of the stable Zn-Al HIM on the limed soil—indicating that Zn^{2+} and Al^{3+} had precipitated out of solution to form the layered mineral and were no longer available, thus contributing to the large decreases in available Zn and Al observed for the limed soil (Table 4.1).

For the SVN site on the other hand, changes in soil pH and in the concentrations of exchangeable Ca and Mg in response to liming were much smaller than at the KN site, suggesting that either: (i) dissolution of the dolostone in the soil was very low and/or (ii) base and metal cations were leaching from the soil to Second Valley in the outwash plain. That is, in addition to the low base cation concentrations in the soil, plant growth at SVN is likely limited by metal (Zn) toxicity and poor nutrient availability (particularly NO_3^-). Moreover, the dense colony of *A. capillaris* on site is also inhibiting the establishment of boreal forest species, as noted during sampling (see Appendix A). Likewise, Winterhalder (1996) noted that the establishment of metal-tolerant *Agrostis* species at the Sudbury mine site was considered a significant barrier to the establishment of other species. Indeed, these *Agrostis* species produce dense rhizomes and sod mats on the surface of the soil that inhibit the germination of other plant species (Garry Oak Ecosystems Recovery Team, 2012). Moreover, the sod mats can become thick enough to smother new growth of the *Agrostis* species themselves in certain years (Winterhalder, 1996). For Zn speciation, Hamilton (unpublished data, 2012) found that liming had less of an effect on Zn speciation at SVN compared to KN. In the non-limed soil at SVN the Zn was predominantly aqueous Zn^{2+} (47%), with secondary concentrations of franklinite (33%) and adsorbed Zn (SiO_2; 20%) at a pH of 4.3; in the limed surface soil, 42% of the Zn was in the form of Zn-layered double hydroxides, with 36% in the form of adsorbed Zn (Zn-MnO_2) and 22% in the form of franklinite at a pH of 6.06. In general, the Zn species formed following liming at the SVN site are more available for plant uptake—reflecting, in part, that the Zn-Al-HIM did not form in the limed SVN soils, likely due to the low concentration of available Al^{3+} (see Table 4.1). Thus, at SVN, it is likely that fertilizer applications together with the removal of *A. capillaris* or seeding/tree planting of native boreal forest species will be required to aid in the re-

establishment of boreal forest species at this site to overcome the Zn toxicity and restricted germination.

4.2 Effects of Soil Type on Plant Biomass Production, Heavy Metal Uptake, and Low Molecular Weight Organic Acids Production

4.2.1 *Silene vulgaris*

4.2.1.1 Plant biomass. There was significantly less growth of *S. vulgaris* in the non-limed soils compared to the reference and limed soils (Fig.4.1). Indeed, both root and shoot biomass production by plants grown in the non-limed soils was significantly ($P < 0.10$) lower than that of plants grown in the limed or reference soils (Fig. 4.2). These results reflect the fact that the transplanted seedlings survived for only 2 wks in the non-limed KN soil and for only 4 wks in the non-limed SVN soil. Consequently, root and shoot biomass production in the non-limed KN soil was only 4.8% and 2.0%, respectively, of the reference. Likewise, root and shoot biomass production in the non-limed SVN soil was 11.0% and 2.0%, respectively, of the reference. These results were somewhat surprising given that the *S. vulgaris* ecotype used in this study—obtained from the Imsbach mine in Germany—was reported to be highly tolerant of Cu, Zn, and Cd (Schat and Vooijs, 1997). The total concentrations of Cu, Zn, and Cd in the Imsbach soil (0-15 cm) were measured in aqua regia digests at 7100, 80, and <0.1 mg kg^{-1} respectively (Schat et al., 1996), however the Flin Flon soils were higher in Zn and Cd, but lower in Cu. Moreover, Song et al. (2004) found *S. vulgaris* established and grew well on soil with 352 mg kg^{-1} of 1*M* NH_4NO_3–extractable Cu, which is higher than both the SVN and KN sites with 52 and 102 mg kg^{-1} of 0.01*M* $CaCl_2$ extractable Cu, respectively. The 1*M* $NH_4NO_3^-$ and 0.01*M* $CaCl_2$ soil extracts for Cu^{2+} may not be directly comparable; however, neutral salt solutions produce the strongest correlations between available metals and shoot metals compared to complexing agents such as DTPA or EDTA, or acids like HCl or Mehlich 1 (Menzies et al., 2007). For Zn, Schat and Vooijs (1997) found *S. vulgaris* required 153 mg L^{-1} $ZnSO_4$ in hydroponic solutions to completely arrest root growth; however, Zn tolerance in *S. vulgaris* (Imsbach) has not been studied in soils with varying concentrations of available Zn^{2+}. Nonetheless, it is unlikely that the available Cu^{2+} or Zn^{2+} in the SVN and KN soils was singularly responsible for the toxicity

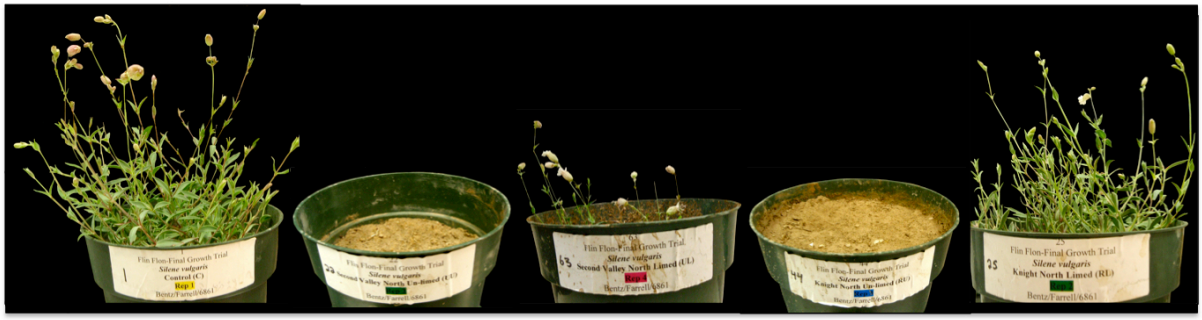

Fig. 4.1. Photographs of *Silene vulgaris* from the growth trial. Soils from left to right are: reference (49 DAP), Second Valley North non-limed (28 DAP), Second Valley North limed (77 DAP), Knight North non-limed (14 DAP), and Knight North limed (49 DAP).

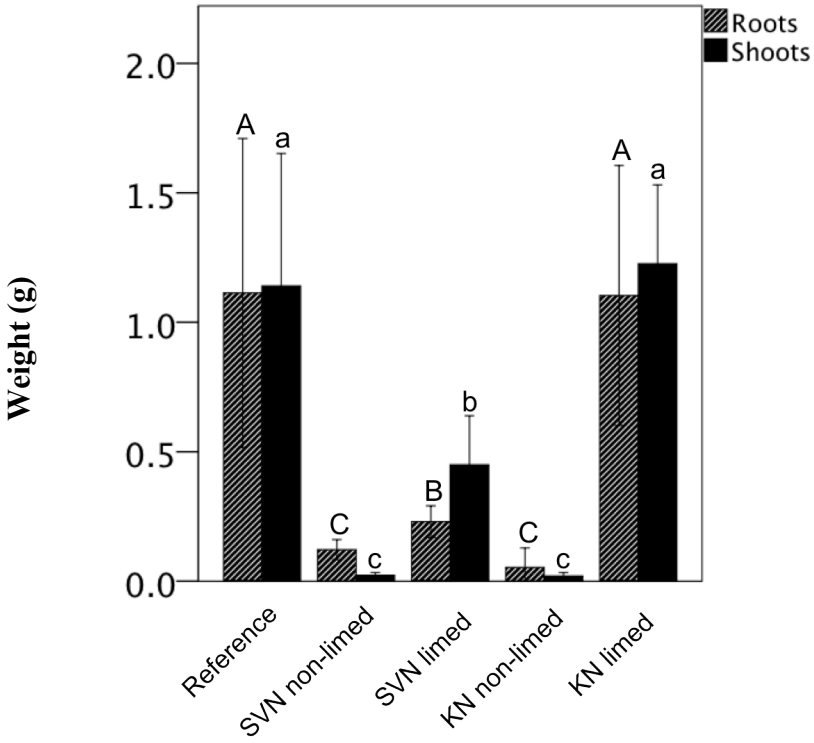

Fig. 4.2. Average weight of the roots (n = 6) and shoots (n = 6) of *Silene vulgaris* grown in soils from the reference (49 DAP), Second Valley North (SVN) non-limed (28 DAP), SVN limed (77 DAP), Knight North (KN) non-limed (14 DAP), and KN limed (49 DAP). Letters above the bar represent significant differences ($P < 0.10$) based on the Kruskal-Wallis test and Games-Howell test. Capital letters apply to the roots while lower case letters apply to the shoots. Error bars represent ± 2 standard errors of the mean.

measured during the growth trial compared to the reference soil. It is more likely that the combination of heavy metals in the soils is controlling toxicity. Whereas metal tolerance studies generally focus on one specific metal, multiple metals are present in high concentrations in the Flin Flon soils; including: Cu, Zn, Cd, Al, and Pb. At SVN, not only did the soils have high concentrations of available Cu^{2+} and Zn^{2+}, they also had the highest concentrations of available Cd^{2+} and $Pb^{2+/4+}$, which may have acted in combination with the Cu^{2+} and Zn^{2+} to produce the observed toxicity. At KN, the available concentrations of Cu^{2+}, Zn^{2+}, Cd^{2+}, and $Pb^{2+/4+}$ in the soil were all higher than the reference soil; but this soil also had a large amount of available Al^{3+} (Table 4.1), which *S. vulgaris* is sensitive to and which was most likely responsible for the very early death of these seedlings.

The root and shoot weights for *S. vulgaris* were also significantly lower ($P < 0.10$) in the non-limed compared to the limed soils for both the SVN and KN sites (Fig. 4.2). Despite the increase in biomass, growth in the SVN soil still resulted in signs of toxicity. Root and shoot weights of *S. vulgaris* in the SVN limed soil compared to those in the reference soil averaged about 40% and 19%, respectively, and were substantially lower than those of the biomass produced on the KN soil, which averaged 108%, and 99%, respectively. While the SVN limed soil had lower concentrations of total and available heavy metals compared to the non-limed soil, the plants did not perform as well in this soil as in the reference or KN limed soil. It is possible the available Zn^{2+} or another factor/metal in the soil is contributing to the toxicity of the SVN site that was not measured. As for the KN limed soil, *S. vulgaris* established and grew well on this soil—equal to the reference—which is likely a result of the low available metal concentrations (especially Al^{3+}) and higher pH.

For smelter affected sites, liming alone is ineffective in alleviating metal toxicity where multiple heavy metals are present in high concentrations (Derome, 2000). Derome (2000) added Mg-enriched limestone (similar to dolostone) to scots pine (*Pinus sylvestris*) stands along a southeast transect from the Cu-Ni smelter in Harjavalta, Finland. After 4.5 years, despite a strong increase in Ca, Mg, and base saturation at all of the sites, none of the liming treatments had a significant effect on exchangeable acidity or Al in the organic layer of plots located closest (*ca*. 0.5 km) to the smelter compared to plots at 4 and 8 km from the smelter. Concentrations of exchangeable Cu and Ni also were reduced only slightly (i.e., by 1.5% for both) compared to the un-amended control. The lack of response to liming at 0.5

km was attributed to the high Fe content in the soil and reduced water-holding capacity of the organic layer (Derome, 2000). Both the precipitation of $Fe(OH)_3$—neutralizing the bicarbonate and hydroxyl ions in the soil—as well as the high flux of water out of the soil—thus drying the surface—were attributed to the reduced reactivity of the limestone (Derome, 2000). Likewise, at SVN the lower CEC, courser texture, lower TOC, higher available Zn^{2+}, and large drainage basin of Second Valley acted in aggregate to decrease the water holding capacity of this soil and subsequently the reactivity of the dolostone.

For this study, it was difficult to discern which of the heavy metals or soil properties measured were responsible for the poor growth in the non-limed soils given the possible synergistic and/or antagonistic interactions between the multiple heavy metals present. Winterhalder (1996) found that Cu and Ni together enhanced each other's phytotoxic effects on redtop (*Agrostis gigantean*) in Sudbury soils, but that Al protected the plants from increased Ni uptake. An et al. (2004) found that when metals were added to sandy loam soil as chloride salts, the toxicity of the metals on cucumber (*Cucumis sativus)* decreased with Cu + Cd and Cu + Pb, but increased with Cd + Pb compared to individual metals at the same concentrations. Moreover, Luo and Rimmer (1995) found that adding $CuCl_2$ to sandy loam soils increased the uptake of Zn added to the soil on barley (*Hordeum vulgare*) increasing toxicity. From these studies it is clear that these interactions are metal and plant specific and the combined effects of Cu, Zn, Cd, Al, Pb, and other metals present in the Flin Flon soils on *S. vulgaris* is not known. Whether specific synergistic or antagonistic effects are present, they are masked by the overall toxicity on the non-limed soils and all of the available metals were negatively correlated with shoot biomass (r = -0.563, $P < 0.01$, r = -0.582, $P < 0.01$; r = -0.691 $P < 0.01$; and r = -0.634 $P < 0.01$ for Cu, Zn, Cd, and Al respectively), with Cd being the most toxic.

4.2.1.2 Metal uptake. Metal uptake in *S. vulgaris* was influenced by the available fraction in the soil. Concentrations of Cu, Zn, and Cd (but not Al) in the roots were correlated with the available metals in the soil (r = 0.668, $P < 0.01$; r = 0.912, $P < 0.01$; and r = 0.693 $P <$ 0.01, respectively). Concentrations of all four metals in the shoots, however, were correlated with the available metals in the soil (Cu: r = 0.684, $P < 0.01$; Zn: r = 0.804, $P < 0.01$; Cd: r = 0.856, $P < 0.01$; and Al: r = 0.825, $P < 0.01$), again indicating the importance of metal availability. For a known excluder, the plant tissues of *S. vulgaris* contained high metal

concentrations, especially the plants grown in the non-limed soils where the biomass was significantly smaller (Fig. 4.3).

For plants grown in the SVN non-limed soils, both the roots and shoots of *S. vulgaris* contained significantly ($P < 0.10$) more Cu, Zn, and Cd compared to those grown in the reference soil. Only in the shoots was the Al concentration greater than the reference, while Al concentrations in the roots were similar. This latter result is not surprising given that the SVN non-limed soil had a similar available Al^{3+} content to the reference soil. The high shoot metal concentrations for *S. vulgaris* in SVN non-limed soil are likely a breakdown of the shoot exclusion mechanisms in the plant given the plants died only 4 wks after transplantation. Compared to the reference soil, the shoot metal concentrations increased by 256×, 319×, 170×, and 29× for Cu, Zn, Cd, and Al, respectively. Excluders like *S. vulgaris,* are able to avoid heavy metal uptake into the shoots of the plant by increasing the pH in the rhizosphere, chelating metals via the root exudates, or through high rates of efflux out of the roots (Greger, 2004). However, when the metal concentrations become too high, crossing the threshold of tolerance, such as in the non-limed soils, the plants lose their ability to avoid uptake due to metal toxicity. Once the function of this barrier is lost, the plants experience massive and uncontrolled metal uptake (Greger, 2004), accounting for the high metal concentrations in the shoots.

Roots of the plants grown in the KN non-limed soil had Cu, Zn, and Al concentrations that exceeded those grown on the reference soil—again reflecting the higher concentrations of available metals in the KN non-limed soil. For the shoots however, plants grown in the non-limed KN soil died within 14 days of being transplanted into the soil; consequently, the amount of metals transported into the shoots was highly variable (see Fig. 4.3). Thus, despite the fact that Cu, Zn, Cd, and Al concentrations in the shoots of plants grown in the KN non-limed soils were 154×, 39×, 145×, and 52× greater than those of plants grown in the reference soil, the differences were not statistically significant.

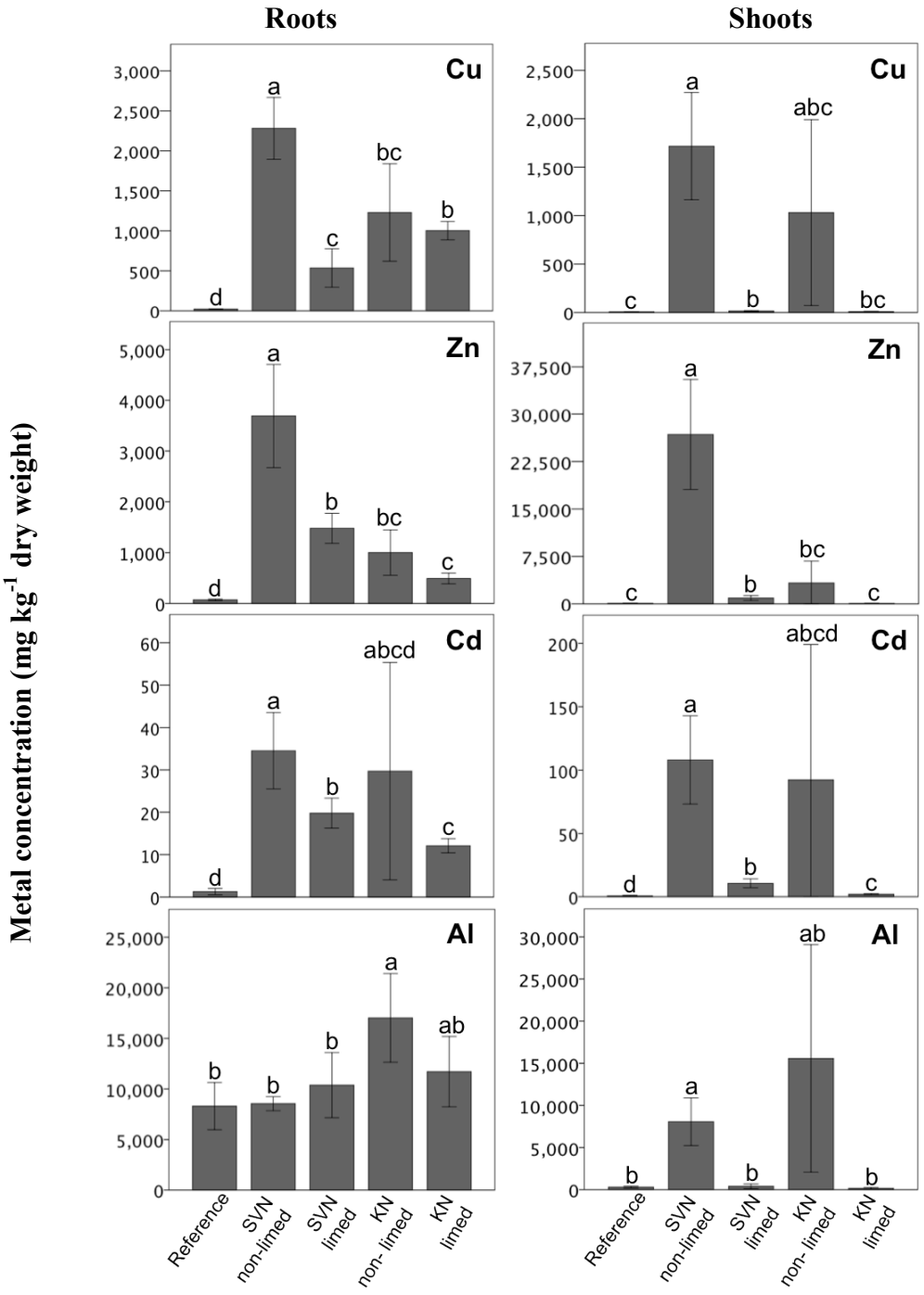

Fig. 4.3. Concentration of heavy metals (mg kg^{-1} dry weight) in the roots (n = 6) and shoots (n = 6) of *Silene vulgaris*. Letters above the bar represent significant differences (P < 0.10) based on the Kruskal-Wallis test and Games-Howell test. Error bars represent ± 2 standard errors of the mean. Note the scale differences on the y-axes between the roots and shoots.

In general, liming resulted in decreased metal concentrations in the shoots of *S. vulgaris* (Fig. 4.3). Compared to plants grown in the non-limed soil, those grown in the SVN limed soil had significantly ($P < 0.10$) lower root concentrations of Cu (23.5%), Zn (40.0%) and Cd (57.3%). Likewise, these plants had significantly lower shoot concentrations of Cu (0.86%), Zn (3.45%), Cd (9.93%), and Al (4.90%) compared to plants grown in the SVN non-limed soil. For the KN site, liming reduced the Cu, Zn, Cd, and Al concentrations in the roots compared to those in the non-limed soils by 18%, 51%, 59%, and 31%, respectively; liming also reduced the shoot metal concentrations compared to the plants grown in the non-limed soil by 91–99%. Nevertheless, the high variability associated with metal uptake by plants grown in the non-limed soil masked the significance of differences in metal concentrations in the roots or shoots. The higher metal concentrations in *S. vulgaris* in the SVN limed soil indicate that some level of toxicity still exists in this soil; i.e., the mechanisms of exclusion are not keeping the heavy metals out of the shoots. Most of the work to date on *S. vulgaris* has focused on genetic mechanisms of Cu tolerance, and shoot metal concentrations for this ecotype (i.e., from the Imsbach population) grown in multi-metal contaminated soil have only rarely been determined. Song et al. (2004) determined Cu concentrations in the shoots of *S. vulgaris* from 30 sites with varying levels of contamination and found the concentrations to range from 3 – 262 mg kg^{-1}, which is higher than the average shoot concentrations from the reference soil (0.635 mg kg^{-1}), but in range for the shoot concentrations from the limed soils at SVN (14.8 mg kg^{-1}) and KN (9.17 mg kg^{-1}). In the limed soils, high concentrations of Cu and Zn in the *S. vulgaris* roots, together with low concentrations in the shoots, indicate that transport of these metals from the roots to the shoots is inhibited—as would be expected for a healthy excluder plant. For the non-limed soils, however, Cu concentrations in the shoots of *S. vulgaris* grown in the Flin Flon soils were much higher than those reported by Song et al. (2004), averaging 1716 mg kg^{-1} for plants grown in the SVN soil and 1031 mg kg^{-1} for plants grown in the KN soil. These excessively high concentrations in the shoots of plants grown in the non-limed soils are a combined result of the short life of the plants (i.e., low shoot weights) and the loss of the root exclusion function. In contrast, for all the soils studied by Song et al. (2004), *S. vulgaris* grew well, displaying no physiological signs of metal stress, indicating that exclusion of metals

into the shoots of the plants was functioning properly, unlike the plants in the non-limed Flin Flon soils.

4.2.1.3 Low molecular weight organic acids (LMWOA). Overall, the highest concentrations of LMWOA (on a per gram dry weight basis) occurred in the non-limed soils (Fig. 4.4), presumably as a response to metal stress and reflecting limited root biomass production and/or the loss of membrane integrity of the root cells. The LMWOA data was highly variable, and significant differences were found for only a few key measurements. For example, *S. vulgaris* grown in the SVN non-limed soil released significantly ($P < 0.10$) more oxalic acid than plants grown in the reference soil and significantly more oxalic and fumaric acid than plants grown in the SVN limed soil. Plants grown in the KN non-limed soil released significantly ($P < 0.10$) more fumaric acid than plants grown in the reference soil or KN limed soil. Positive correlations between the concentrations of oxalic acid and available Cu^{2+}, Zn^{2+}, Cd^{2+}, and Al^{3+} in the soils were significant ($P < 0.05$) suggesting the metals may stimulate oxalic acid release. Harmens et al. (1994) examined the detoxification mechanisms of heavy metals in *S. vulgaris*, with a focus on organic acid chelates within the shoots, roots, and xylem and found no conclusive evidence linking malic, citric, oxalic, or succinic acids to increased tolerance. They did however note though that *S. vulgaris* produced high amounts of oxalic acid naturally, which is consistent with the results obtained in the present study (Fig. 4.4). Therefore, it appears that the high concentration of oxalic acid found in the soils, particularly the non-limed soils, may be a result of a metal tolerance strategy to inhibit the uptake of heavy metals. Lastly, there was no statistical difference between specific organic acids in the reference soil, the SVN limed soil, and the KN limed soil suggesting the increased release of organic acids in the non-limed soils is a result of metal stress and/or cell lyses.

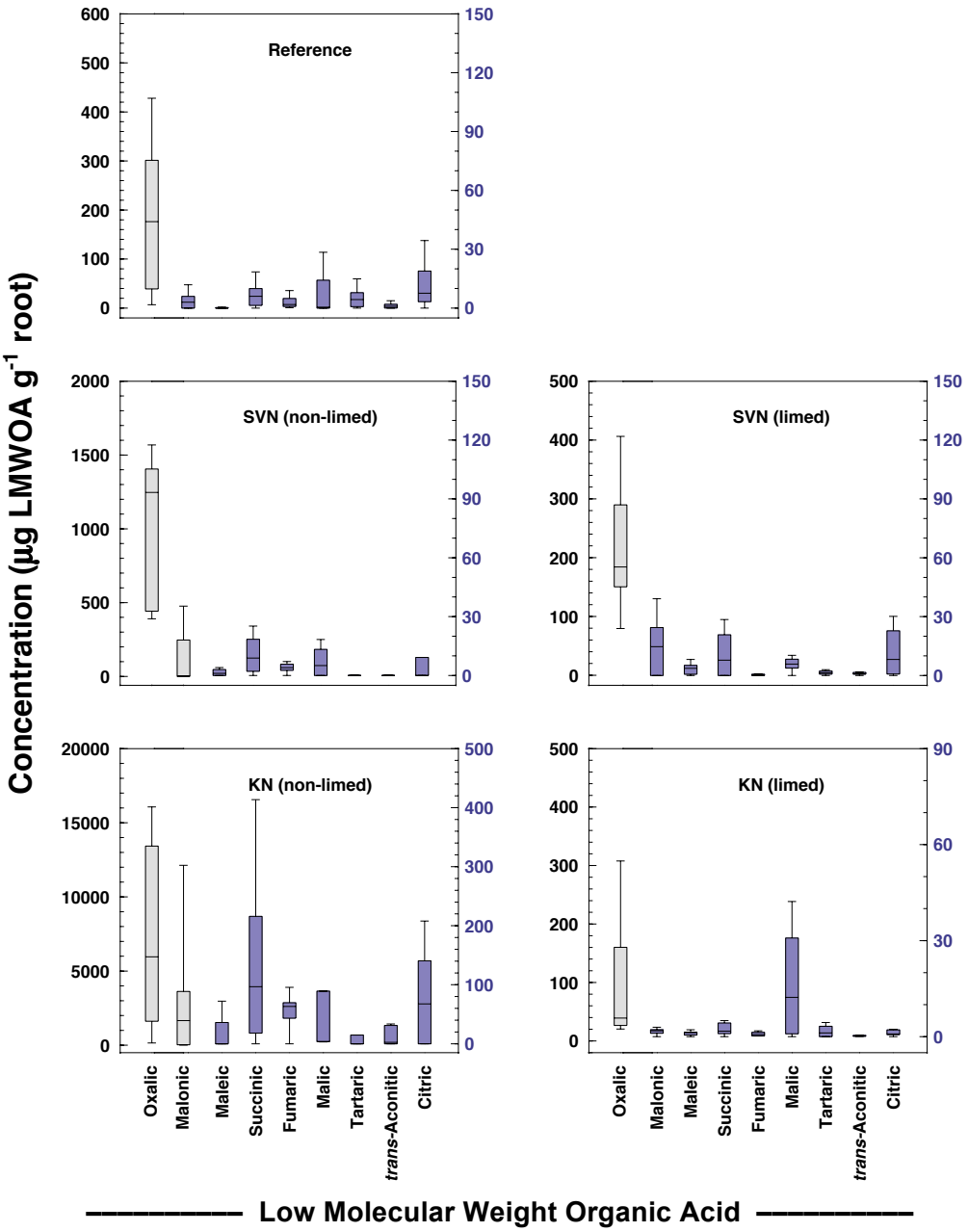

Fig. 4.4. Box and whisker plots of the low molecular weight organic acids (LMWOA) released into the soil by *Silene vulgaris* (n = 6) growing on the reference, Second Valley North (SVN) non-limed and limed, and Knight North (KN) non-limed and limed soils. Note: where applicable, the right-hand y-axis corresponds to the blue box & whisker bars. The data median (central tendency line), 1st and 3rd quartiles (bottom and top of box) and minimum and maximum values (bottom and top of whiskers) are displayed.

4.2.2 *Agrostis capillaris*

4.2.2.1 Plant biomass. In general, *A. capillaris* grew better (Fig. 4.5) and produced more biomass (Fig. 4.6) than *S. vulgaris* (see Figs. 4.1 and 4.2); nevertheless, there was significantly less growth of *Agrostis capillaris* in the non-limed KN soil than in any of the other soils (Fig. 4.5). Not surprisingly, root and shoot weights of plants grown in the non-limed KN soil were significantly ($P < 0.10$) lower than those of the other plant/soil combinations (Fig. 4.6). The lone exception being the roots of plants grown in the KN limed soil (Fig. 4.6). Plants grown in the SVN soils (limed and non-limed) yielded biomass production (above- and below-ground) that was comparable to that of plants grown in the reference soil. However, plants grown in the SVN non-limed soil showed signs of chlorosis and necrosis (see Fig. 4.5). As well, plants grown in the KN non-limed soils were characterized by stunted, dark green leaves (see Fig. 4.5). These observations are consistent with the work of Shaw et al. (2004) who compiled visual toxicity symptoms in response to heavy metal stress. They reported chlorosis caused by Zn and Cd, necrosis caused by Cd, stunting caused by Zn, and increased shoot production caused by Pb. Shaw et al. (2004) also noted that Al can cause the production of stunted, dark green leaves and a reduction in root growth.

Given that *A. capillaris* was found growing on the SVN soils in Flin Flon, the good growth of this plant on these soils during the greenhouse study is not surprising. Documented cases of metal tolerance for certain sites exist for *A. tenuis* (a synonymous species to *A. capillaris)* with Zn, Cd, and Pb in Northern France; *A. scabra* for Cu and Ni in Sudbury ON; and *A. capillaris* for Zn, Cd, and Pb in East Belgium—supporting the theory that metal tolerance is specific to individuals or populations, not whole species, depending on the environmental conditions and selective pressure of the heavy metals (Archambault and Winterhalder, 1995; Dahmani-Muller et al., 2000; Meerts and Grommesch 2001). Wide ranging investigations of metal tolerances for *A. tenuis (capillaris)* across mining sites in Belgium found that this plant has the ability to evolve tolerances to uncommon heavy metals, each of which are separate and specific to a particular metal (Gregory and Bradshaw, 1964).

The ecotype of *A. capillaris* growing at the SVN site in Flin Flon developed its tolerance to the specific set of physical and chemical conditions present in that soil, thus

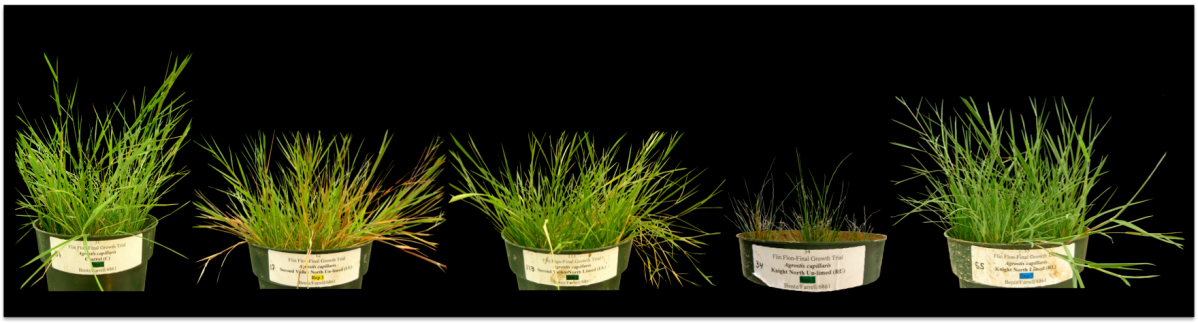

Fig. 4.5. Photographs of *Agrostis capillaris* from the growth trial. Soils from left to right are: reference (49 DAP), Second Valley North non-limed (63 DAP), Second Valley North limed (49 DAP), Knight North non-limed (84 DAP), and Knight North limed (63 DAP).

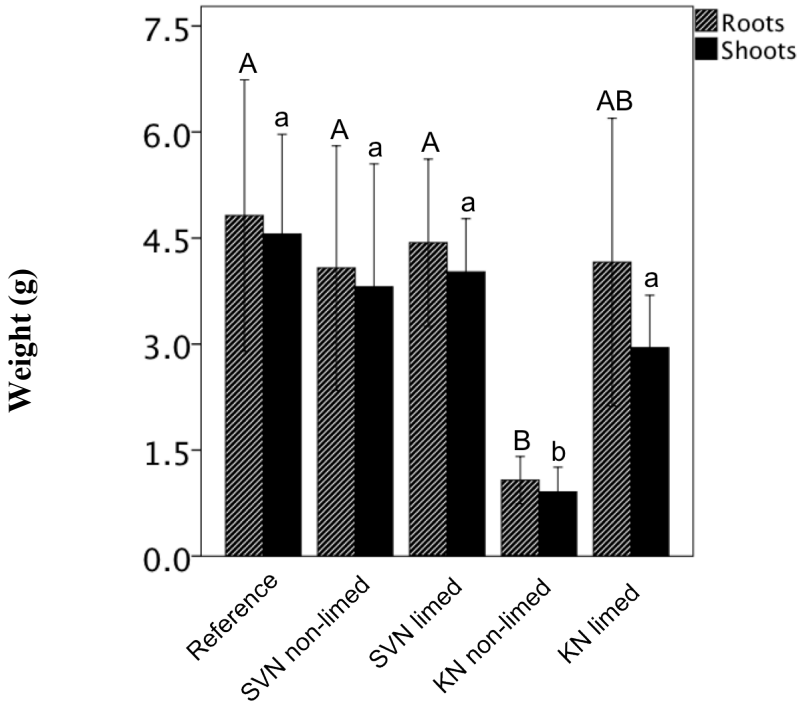

Fig. 4.6. Average weight of the roots (n = 6) and shoots (n = 6) of *Agrostis capillaris* grown in soils from the reference (49 DAP), Second Valley North non-limed (63 DAP), Second Valley North limed (49 DAP), Knight North non-limed (84 DAP), and Knight North limed (63 DAP). Letters above the bar represent significant differences ($P < 0.10$) based on the Kruskal-Wallis test and Games-Howell test. Capital letters apply to the roots while lower case letters apply to the shoots. Error bars represent ± 2 standard errors of the mean.

explaining the apparent lack of response to lime in the field. That is, vegetative growth is likely stunted due to nutrient deficiencies as has been seen at other metal enriched sites (Redente and Richards, 1997; Córdova et al., 2011; Ulriksen et al., 2012). In the greenhouse study however, the plants were watered with nutrient solution until they became established, thus removing (or at the very least, minimizing) nutrient deficiencies characteristic of the field soils. The result is a much healthier plant stand in the greenhouse compared to the SVN field site (Appendix A; Fig. A2). Root and shoot biomass production by plants grown in the SVN non-limed soil compared to those in the reference soil were 85% and 84%, respectively. For plants grown in the SVN limed soil, root and shoot biomasses compared to those in the reference soil were 92% and 88%, respectively. These data suggest that water and nutrients, not metal toxicity, are the primary factors limiting plant growth and the revegetation potential of the SVN site.

In Sudbury ON, Archambault and Winterhalder (1995) found that *A. scabra* colonized barren, contaminated areas after fertilization and irrespective of lime applications. Additional metal tolerance studies (under hydroponic conditions) found that plants from the contaminated sites grew better in the presence of heavy metals than in their absence, indicating that the plants had developed a physiological dependence on the heavy metals present in the soil (Archambault and Winterhalder, 1995). This physiological dependence for heavy metals may also hold true for the *A. capillaris* seeds collected from the SVN site, as these plants generally grew better in the SVN soil than KN soil. Plants grown in the KN non-limed soil yielded root and shoot weights of those in the reference soil at 22% and 20%, respectively; the root and shoot weights of plants grown in the limed soil to those in the reference soil were at 86% and 65%, respectively. In relation to the soils data, biomass production by *A. capillaris* was negatively correlated with available Cu^{2+} (r = -0.540 $P <$ 0.01; r = -0.557, $P < 0.01$ for the roots and shoots, respectively) and Al^{3+} (r = -0.447 $P <$ 0.05; r = -0.405, $P < 0.05$ for the roots and shoots, respectively).

4.2.2.2 Metal uptake. *Agrostis capillaris* is a typical metal excluder that, like *S. vulgaris* when healthy, immobilizes metals in the below ground biomass of the plant, yielding higher metal concentrations in roots compared to the shoots (Dahmani-Muller, 2000). Indeed, data from the current study show that plants grown in the SVN and KN soils have greater

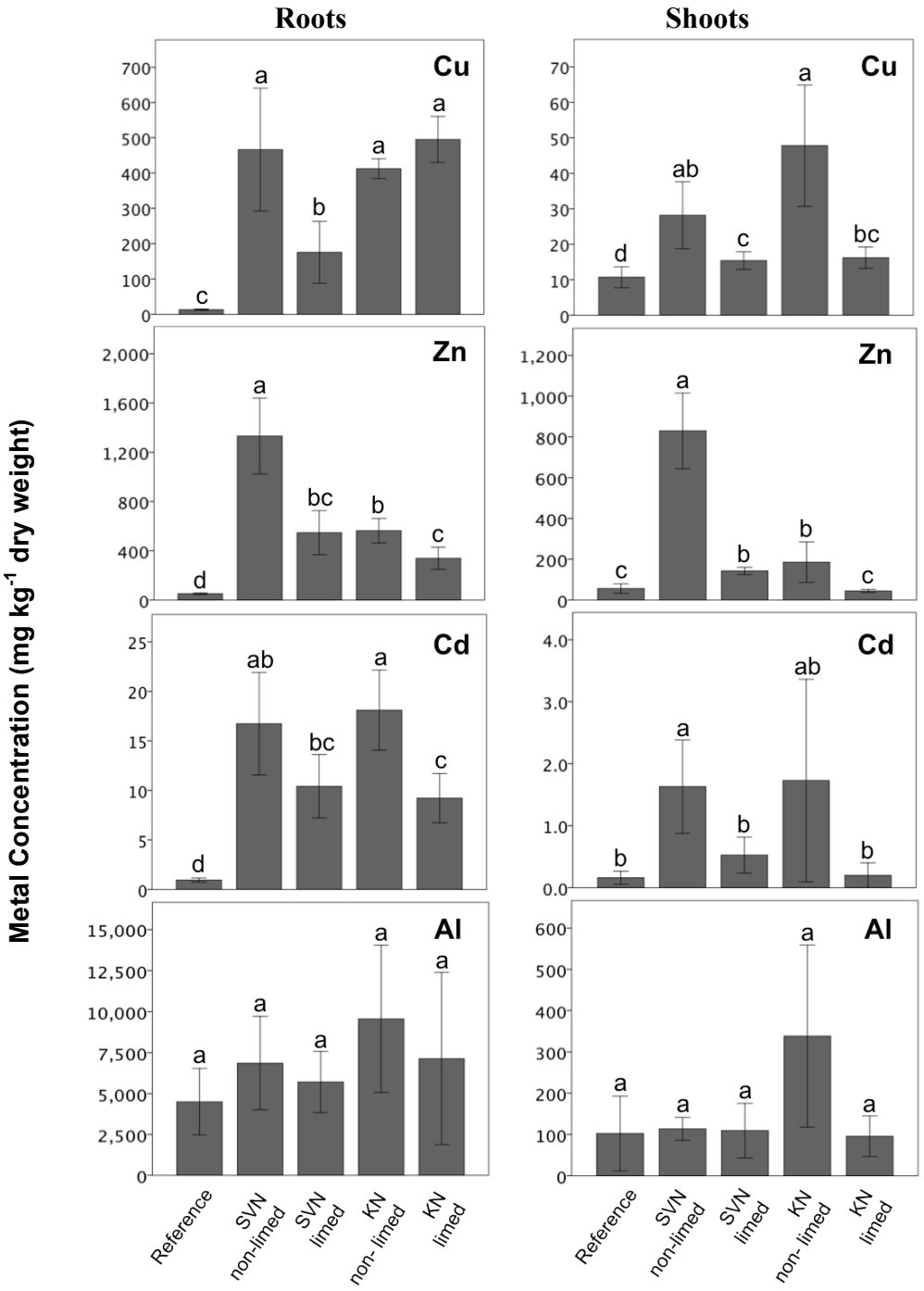

Fig. 4.7. Concentration of heavy metals (mg kg^{-1} dry weight) in the roots (n = 6) and shoots (n = 6) of *Agrostis capillaris*. Letters above the bar represent significant differences ($P < 0.10$) based on the Kruskal-Wallis test and Games-Howell test. Error bars represent ± 2 standard errors of the mean. Note the scale differences on the y-axes between the roots and shoots.

concentrations of metals in the roots than in the shoots (Fig. 4.7), indicating metal exclusion from the shoots. This is especially true in the limed soils (Fig. 4.7). However, the threshold concentrations above which the exclusion mechanism for each metal becomes inactive are not known and higher concentrations of heavy metals generally were found in the shoots of the plants grown in the non-limed soils.

For the non-limed soils, the plant roots and shoots both contained higher concentrations of the smelter-related metals compared to plants grown in the reference soil—reflecting the availability of the metals present in the soils. Specifically, the roots of *A. capillaris* grown in the SVN non-limed soil contained significantly ($P < 0.10$) more Cu (35×), Zn (26×), and Cd (17×) than did the roots of plants grown in the reference soil, which corresponds with the higher concentrations of available metals in these soils. Whereas the shoots of plants grown in the SVN non-limed soil also contained more ($P < 0.10$) Cu (14×), Zn (15×), and Cd (10×) than did the shoots of plants grown in the reference soil, increases in the shoot concentrations were considerably smaller than those in the roots. For Al, differences in the root and shoot concentrations between the SVN non-limed and reference soils were generally small reflecting the similarity in available Al concentrations in these soils.

In the KN non-limed soil, the roots of *A. capillaris* contained significantly ($P < 0.10$) higher concentrations of Cu (31×), Zn (11×), and Cd (19×) compared to plants grown in the reference soil (Fig. 4.7). However, unlike the plants grown in the SVN soils, metal concentrations in the shoots of plants grown in the KN non-limed soil were greater only for Cu (4.5×; $P < 0.10$) and Zn (3.3×; $P < 0.10$) when compared to the reference, despite obvious signs of metal toxicity (see Fig. 4.5). The shoot Cd concentrations in the KN non-limed soil were highly variable (averaging 11× those in the reference soil), and the soil may have had Cd^{2+} concentrations near the threshold of tolerance; thus contributing to the observed variability. Relative to the reference soil, root and shoot Cd concentrations were much lower in *A. capillaris* than in *S. vulgaris*, which presumably reflects the greater Cd tolerance (and, hence survival) of *A. capillaris.*

In general, root and shoot metal concentrations in *A. capillaris* declined in response to liming. In the SVN soil, the roots of plants grown in the limed soil compared to those in the non-limed soil had significantly ($P < 0.10$) lower concentrations of Cu (38%) and Zn (41%),

which again corresponds to the lower concentrations of available Cu^{2+} and Zn^{2+} in the limed soil. Liming had no significant effect on root Cd concentrations in plants grown in the SVN soil, despite a small decrease in the amount of available Cd^{2+} in the soil (i.e., from 3.22 to 1.57 mg kg^{-1}). The high Zn concentrations in both the non-limed and limed soil could have interfered with Cd uptake. Indeed, Lombi et al. (2001) found that in hydroponic systems high concentrations of Zn relative to Cd significantly decreased the uptake of Cd in the hyperaccumulator *T. caerulescens*. Moreover, Lasat et al. (2000) found that the Zn transporter ZNT1 also mediated low affinity Cd transport. On the other hand, liming the SVN soil resulted in significantly reduced shoot Zn concentrations (Fig. 4.7), which corresponded with lime-induced decreases in concentrations of both the available Zn^{2+} in the soil and roots. Copper and Cd concentrations in the shoots also tended to decrease in response to liming compared to those in the reference soil, averaging 55% and 32%, respectively (Fig. 4.7), though these differences were not as drastic due to the controlled translocation of Cu in plants and the lower uptake of Cd into the roots.

For plants grown in the KN soil, the effects of liming on root and shoot metal concentrations were more complex. Liming significantly ($P < 0.10$) reduced the root concentrations compared to those in the non-lime soil for Zn (71%) and Cd (51%), but increased the root concentration of Cu (Fig. 4.7). In fact, the average root Cu concentration in the limed soil was 20% higher than in the non-limed soil, indicating accumulation of the metal in the roots of healthy plants. Given the low concentration of available Cu^{2+} in the limed soil, it is interesting to note that there was such a large accumulation of Cu in the roots. On the other hand, shoot concentrations of Cu, Zn and Cd decreased in response to liming—with Cu showing the largest decrease (-34%), despite an increase in the total concentration of Cu in the roots (Fig. 4.7). Presumably, healthy plants on the limed soil effectively inhibited the translocation of Cu into the shoots—averaging only 16.2 mg kg^{-1}, which is within the normal range of shoot Cu concentrations (i.e., 2–20 mg kg^{-1}; Hagenmeyer, 2004). For Cd, highly variable shoot concentrations in plants grown in the non-limed soil (Fig. 4.7) likely contributed to the lack of statistical significance when compared to plants grown in the limed soil, indicating that the concentration of available Cd^{2+} in the soil is near the threshold of tolerance—even though liming decreased the mean Cd concentration by 89%. Similar results were observed for *S. vulgaris*.

For *A. capillaris*, liming had no significant effect on root or shoot concentrations of Al in either the SVN or KN soils (Fig. 4.7). Moreover, Al concentrations in the roots and shoots of plants grown in the reference soil were about the same as those of the plants grown in the smelter-impacted soils (Fig. 4.7), which may be due to different physiological mechanisms of metal tolerance. Miyasaka et al. (1991) found that Al-tolerant cultivars of *Phaseolus vulgaris* excreted 70 times more citric acid in Al stressed hydroponic systems than Al-sensitive cultivars, leading to the hypothesis that Al resistance is a result of the formation of Al-citrate complexes and the associated decrease in activity of Al^{3+} around the roots. The increased exudation of organic acids under Al stress also was found with oxalate in buckwheat, citrate in maize, and malate in wheat (Zheng et al., 1998; Mariano and Keltjens, 2003; and Sasaki et al., 2004). For other metals that are essential micronutrients (e.g., Zn, Cu, and Ni) required for metabolic functioning, or are chemical analogs of a micronutrient element (e.g., Pb and Cd), complete exclusion from the root cells is not possible (Prasad, 2004). Instead, heavy metals other than Al need to be sequestered and detoxified within the cells of the plants (Prasad, 2004), making the thresholds of tolerance more important. For example, Turner and Marshall (1972) studied Zn sequestration in the roots of *A. capillaris* and concluded that Zn-tolerant plants were able to accumulate Zn in the cell wall with an altered carbohydrate, effectively preventing Zn from entering the cell. However, as with other excluders, when the Zn adsorption sites on the cell wall become saturated, Zn transport throughout the plant occurs, thus exceeding the threshold of tolerance (Turner and Marshall, 1972), which is likely what was observed for the plants grown in the SVN non-limed soils. In connection with differences in metal tolerance strategy, metal concentrations in the roots and shoots of *A. capillaris* were correlated with the amounts of available metal in the soil; that is, except for Al. Concentrations of Cu, Zn, and Cd in the roots were significantly ($P < 0.01$) correlated with the available metal concentrations ($r = 0.495$, 0.896 and 0.691, respectively). However, as was observed for *S. vulgaris*, there was no correlation between the concentrations of available Al in the soil and the roots. For the shoot metal concentrations, the correlations were statistically significant for all of the metals ($r = 0.817$, 0.807, and 0.677 for Cu, Zn, and Cd, respectively) including Al ($r = 0.392$, $P < 0.05$), though the Al correlation was by far the weakest—accounting for only 15% of the variability in the data.

4.2.2.3 Low molecular weight organic acids (LMWOA). As was the case with *S. vulgaris,* the LMWOA concentrations in soil growing *A. capillaris* were highly variable (Fig. 4.8), and there were few significant differences between soils. *Trans*-aconitic acid was present in relatively high concentrations in the reference and SVN soils, but was barely detectable in the KN soils. Moreover, liming tended to increase ($P = 0.11$) the release of *trans*-aconitic acid in the SVN soil; i.e., *trans*-aconitic acid concentrations increased in the order: SVN non-limed soil < reference soil < SVN limed soil. Recalling that plants grown in the SVN non-limed soil showed signs of metal-induced chlorosis (see Fig. 4.5), this data suggests that *trans*-aconitic acid is a product of healthy plants. Indeed, Cawthray (2003) investigated LMWOAs in rhizosphere extracts from field pea (*Pisum sativum*), *Banksia attenuata*, white lupin (*Lupin albu*) and chickpea (*Cicer arietinum*) and found that *trans*-aconitic acid was present in the rhizosphere of all four species. Based on these results, Cawthray (2003) speculated that *trans*-aconitic acid (together with several other LMWOAs) played a role in nutrient acquisition. Likewise, Gaume (2001) found that *trans*-aconitic acid (together with malic and citric acids) was involved in P acquisition in maize. The predominant LMWOAs in the rhizosphere of *A. capillaris* grown in the KN soils were oxalic and citric acids (Fig. 4.8). As well, small, but significant, amounts of maleic and succinic acid also were present in these soils (Fig. 4.8), though both acids were present in higher concentrations ($P < 0.10$) in the non-limed soil. This suggests that these acids either were produced in higher quantities in response to a metal/nutrient-induced stress or were released as a result of root damage. Data analysis revealed that there were correlations between the amount of maleic acid found in the soil and the concentrations of available Cu^{2+} and Al^{3+} in the soil ($r = 0.696$, $P < 0.01$ and $r = 0.663$, $P < 0.01$, respectively). Aluminum stress has been shown to increase the exudation of citric, malic, succinic and oxalic acids in Al-tolerant plants (Delhaize et al., 1993; Zheng et al., 1998; Mariano and Keltjens, 2003; and Sasaki et al., 2004), therefore maleic acid may also be involved in alleviating Al toxicity for *A. capillaris*. Thus, given the high concentration of available Al^{3+} in the KN non-limed soil, it seems plausible that the increase in maleic and succinic acids are most likely a result of Al toxicity or root damage. Overall, it appears that soil type and liming had a greater effect on the LMWOA profiles developed under *A. capillaris* than under *S. vulgaris*.

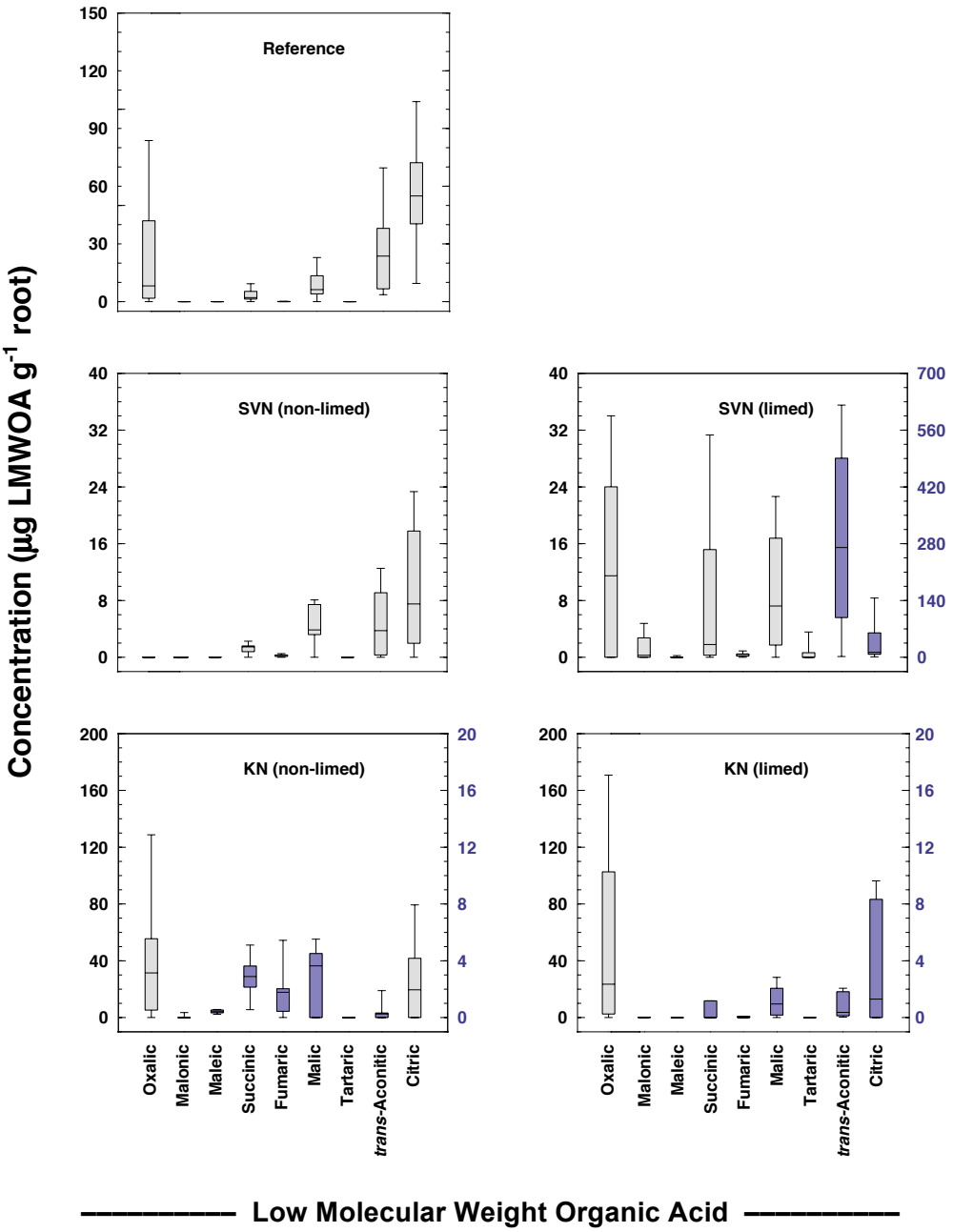

Fig. 4.8. Box and whisker plots of the low molecular weight organic acids (LMWOA) released into the soil by *Agrostis capillaris* (n = 6) growing on the reference, Second Valley North (SVN) non-limed and limed, and Knight North (KN) non-limed and limed soils. Note: where applicable, the right-hand y-axis corresponds to the blue box & whisker bars. The data median (central tendency line), 1st and 3rd quartiles (bottom and top of box) and minimum and maximum values (bottom and top of whiskers) are displayed.

4.2.3 *Pelargonium* sp. 'Frensham'

4.2.3.1 Plant biomass. The growth and survival of *P.* sp. 'Frensham' was reduced on the non-limed soils as the plants suffered from stunted growth, chlorosis, and necrosis of mature leaves (Fig. 4.9). For *P.* sp. 'Frensham', the tissue weights on the non-limed SVN and KN soils were significantly ($P < 0.10$) smaller than those in the reference soil, indicating that the hyperaccumulator also suffered from metal toxicity, or nutrient deficiency, in the non-limed soils (Fig. 4.10). In the SVN non-limed soils, root and shoot weights compared to those in the reference soil were only 18% and 23%, respectively; while in the KN non-limed soils, the root and shoot weights were 21% and 29%, respectively, of those for the plants grown in the reference soil. Nonetheless, *P.* sp. 'Frensham' has been proven to remain metabolically active in hydroponic systems containing single metal concentrations of Cd (500 mg L^{-1}), Ni (500 mg L^{-1}) and Pb (1560 mg L^{-1}) (Dan et al., 2000; KrishnaRaj et al., 2000), however the multiple metals present in Flin Flon have not been tested. Moreover, greenhouse trials with *P.* sp. 'Frensham' by Saxena and KrishnaRaj (1999) demonstrated that the plant also was able to accumulate Cu and Zn from contaminated soils and store the metals in the aboveground foliage. Most of the tolerance testing for *P.* sp. 'Frensham' has involved Cd, Ni and Pb; thus it is possible that (i) Zn in the SVN soil or Cu and Al in the KN soil were above the threshold of tolerance or (ii) the combination of metals found in the non-limed soils acted synergistically to enhance metal-induced stress. Whereas a few plants grown in the SVN non-limed soil (which is high in available Zn, Cd and Pb) died, *P.* sp. 'Frensham' performed slightly better on the KN soils. Given this species demonstrated tolerance to Cd and Pb (Dan et al., 2000; KrishnaRaj et al., 2000), the reduced growth in the SVN soil indicates that the plants may be more sensitive to Zn. However, this perceived sensitivity to Zn is somewhat surprising as both Cd and Zn behave similarly with respect to uptake. Cadmium usually causes toxicity by mimicking Zn and substituting for Zn enzymes such as alcohol dehydrogenase, adenosinetriphosphatase, amylase, and carbonic anhydrase (Shaw et al., 2004). Again, as was the case with *S. vulgaris*, other physical or chemical factors were likely contributing to the poor vigor demonstrated by *P.* sp. 'Frensham'. These factors could include low soil pH or the presence of other heavy metals and metalloids (e.g., As or Hg) in the soil (Bentz and Farrell, unpublished data).

Fig. 4.9. Photographs of *Pelargonium* sp. 'Frensham' from the growth trial. Soils from left to right are: reference (77 DAP), Second Valley North non-limed (63 DAP), Second Valley North limed (77 DAP), Knight North non-limed (77 DAP), and Knight North limed (77 DAP).

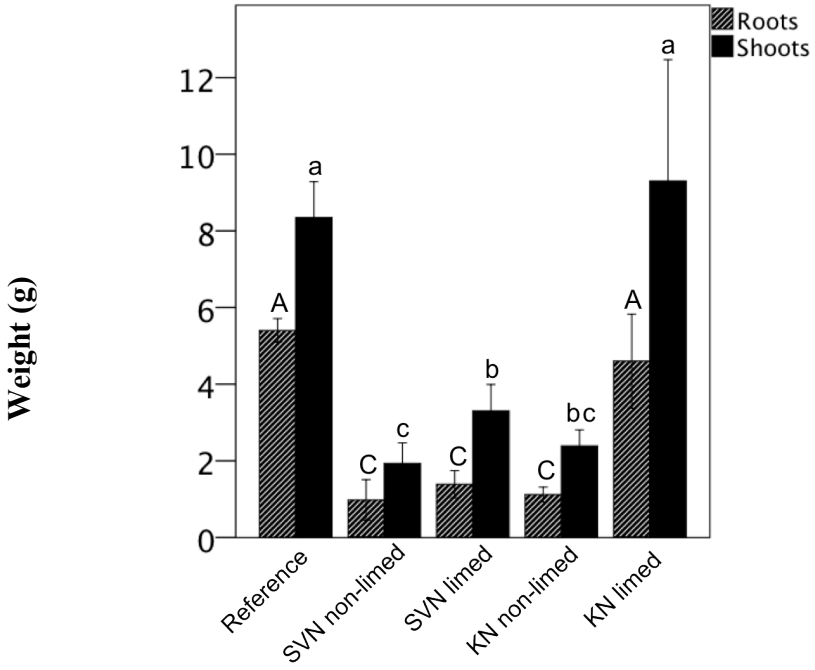

Fig. 4.10. Average weight of the roots (n = 6) and shoots (n = 6) of *Pelargonium* sp. 'Frensham' grown in soils from the reference (77 DAP), Second Valley North (SVN) non-limed (63 DAP), SVN limed (77 DAP), Knight North (KN) non-limed (77 DAP), and KN limed (77 DAP). Letters above the bar represent significant differences ($P < 0.10$) based on the Kruskal-Wallis test and Games-Howell test. Capital letters apply to the roots while lower case letters apply to the shoots. Error bars represent ± 2 standard errors of the mean.

Dolostone applied to the field sites by the *Green Project* increased growth of *P.* sp. 'Frensham' in soils from the SVN and KN sites. In general, the roots and shoots of plants grown in the limed soils were larger than those of plants grown in the non-limed soils, though the difference was not significant ($P < 0.10$) for the roots of plants grown in the SVN soil. Soil pH increased following addition of the dolostone and, in turn, this caused a shift in metal speciation and decreased metal availability (Hamilton, unpublished data, 2012). As a result, the factors contributing to the toxicity of *P.* sp. 'Frensham' on the non-limed soils were, to some extent, relieved by liming—this was especially true for the KN soil.

Compared to plants grown in the reference soil, plants grown in the SVN limed soil suffered from some level of toxicity, likely due to the high concentrations of available Zn^{2+} in the soil, as was evidenced by significantly ($P < 0.10$) smaller root and shoots of the plants in the SVN soil. Tissue weights for the roots and shoots of plants grown in the SVN limed soil to those in the reference soil were 26% and 40%, respectively. On the other hand, growth of the plants in the KN limed soil was equal to or better than that of the plants grown in the reference—with root and shoot weight equal to 85% and 111%, respectively, of the reference. These results provide a clear indication that the dolostone had effectively alleviated the conditions that limited growth in the non-limed soil.

4.2.3.2 Metal uptake. *Pelargonium* sp. 'Frensham' is a known metal accumulator with the ability to concentrate multiple heavy metals in its aboveground biomass (Saxena and KrishnaRaj, 1999). In keeping with its known ability to translocate metals into its shoots (even at low soil concentrations), *P.* sp. 'Frensham' accumulated the highest concentrations of Cu, Zn, Cd, and Al of any of the plants grown in the reference and limed soils. The only exceptions occurred for *S. vulgaris*, which had the highest shoot concentrations of Zn (in the SVN limed soil) and Cd (in the KN limed soil), but which were largely a reflection of the low biomass production associated with these soils. When true bioaccumulation of metals in the aboveground foliage of hyperaccumulators occurs, the shoot concentrations of heavy metals do not linearly correlate with the concentration of available metals in the soil (Zhao et al., 2003). However, for *P.* sp. 'Frensham', both root and shoot metal concentrations were linearly correlated with the available concentrations of the smelter-related metals, indicating that metal availability in the soil accounted for more of the variation in shoot metals than did bioaccumulation. For Cu, Zn and Cd, the available metal concentrations in the soils were

highly correlated ($P < 0.01$) with the root metal concentrations ($r = 0.502$, 0.914 and 0.922, respectively), and were the strongest correlations for Zn and Cd determined for any of the plants tested. Shoot concentrations of Cu, Zn and Cd also were strongly correlated ($P < 0.01$) to the available soil fraction ($r = 0.727$, 0.940 and 0.843, respectively). When comparing hyperaccumulators grown in soils with wide ranging metal fractions, or very high concentrations of available metals (such as in the un-limed soils), the relationship between available metal concentration in the soil and the shoot metal concentration often increases while the bioconcentration factor decreases, likely due to saturation of the metal uptake system (Zhao et al., 2003). Under the conditions in the greenhouse and soils used for this study, *P.* sp. 'Frensham' did not hyperaccumulate any of the smelter-related metals; i.e., shoot metal concentrations were less than 1,000 mg kg^{-1} of Cu; 10,000 mg kg^{-1} of Zn; and 100 mg kg^{-1} of Cd (Fig. 4.11) (Baker and Brooks, 1989). Hydroponic studies with *P.* sp. 'Frensham' have measured shoot Cd concentrations as large as 778 mg kg^{-1} dry tissue after 14 days exposure to 750 mg L^{-1} of $Cd(NO_3)_2$, confirming that the plant can hyperaccumulate Cd (Dan et al., 2002). Thus, data from the present study suggests that a lack of hyperaccumulation by the plants may have been due to toxic effects induced by the high concentrations of Cu, Zn and Al in the soils (and for which the thresholds of tolerance have not been determined). As well, these results may reflect dilution of metals within the plants; as the cuttings transplanted into the soils were fairly large to begin with (i.e., past the two-leaf stage, with lignification of the stems), and metals accumulated in the plants after transplantation were "diluted" into the existing biomass.

For the non-smelter related metal Al, relationships between the available Al^{3+} in the soils, root Al, and shoot Al followed the characteristic patterns of hyperaccumulation. For Al in the roots, *P.* sp. 'Frensham' was the only plant to have a significant correlation to the available soil fraction, though it was negative ($r = -0.409$, $P < 0.05$). For the shoots, *P.* sp. 'Frensham' was the only plant species that did not have a significant correlation between the shoot Al and available soil Al^{3+}. Thus the limed soils produced relatively high foliar concentrations of Al with lower available concentrations in the soil, indicating a substantial transfer of Al to the shoots (Fig. 4.11). Hyperaccumulation of Al has not been as extensively studied as the other smelter-related metals, but does exist. Given that the mean concentration

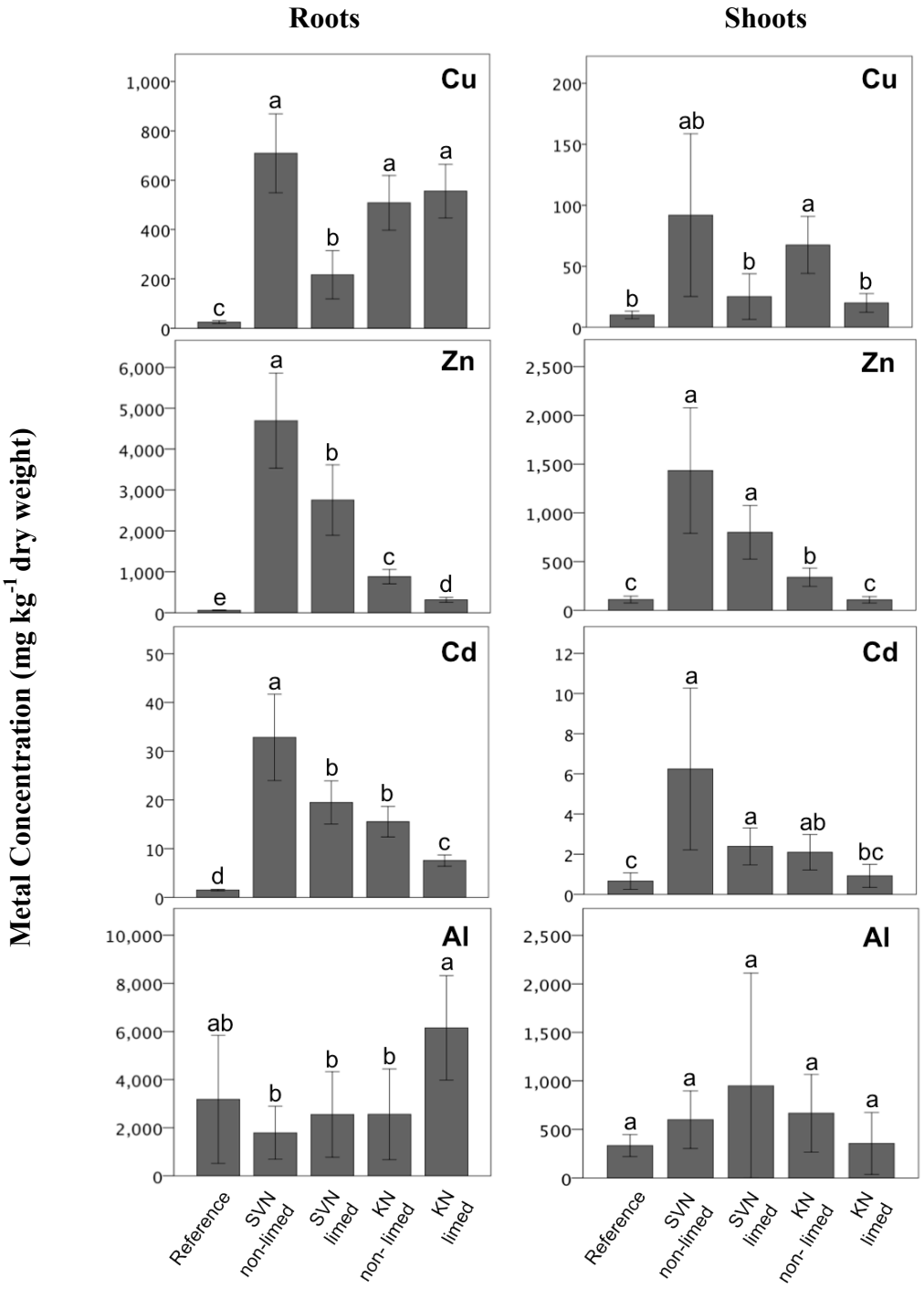

Fig. 4.11. Concentration of heavy metals (mg kg^{-1} dry weight) in the roots (n = 6) and shoots (n = 6) of *Pelargonium* sp. 'Frensham'. Letters above the bar represent significant differences (P < 0.10) based on the Kruskal-Wallis test and Games-Howell test. Error bars represent ± 2 standard errors of the mean. Note the scale differences on the y-axes between the roots and shoots.

of Al in plant tissues is 200 mg kg^{-1} dry leaf weight, Jansen et al. (2002) proposed a threshold value of 1000 mg kg^{-1} dry leaf weight for the hyperaccumulation of Al. Shoots of the *Pelargonium* species used in this study accumulated, on average, 334, 600, 948, 666, and 356 mg Al kg^{-1} dry shoot weight in the reference, SVN non-limed, SVN limed, KN non-limed, and KN limed soils, respectively (Fig. 4.11). On the SVN limed soil, the shoot concentrations of Al neared the 1000 mg kg^{-1} threshold of hyperaccumulation, and given the entire aboveground biomass was combined for analysis in this study, the leaves themselves may have had an even higher concentration of Al. For the other four soils, all of the shoot concentrations were still above the average Al content in plants, making *P*. sp. 'Frensham' an Al accumulator. To date, hyperaccumulation of Al in the Geraniaceae family has not been documented. However, the high shoot Al concentrations, negative correlation between the root Al and available Al, and the lack of a correlation between shoot Al and available Al all indicate an increased uptake of the metal from the soil and translocation to the aboveground biomass, characteristic of metal accumulation.

In reviews of Al hyperaccumulation, it has been noted that the vast majority of hyperaccumulators are woody angiosperms (Jansen et al., 2002) and that tropical species occupying acidic, leached, forest soils have consistently higher Al concentrations in the shoots of the plants compared to temperate species (Metali et al., 2011). According to Jansen et al. (2002) the most commonly studied Al hyperaccumulators are in: the Vochysiaceae family, the Melastomataceae family, and the Lycopodiacea family. However, *Camellia oleifera*, *Richeria grandis*, *Fagopyrum esculentum*, and *Hydrangea macrophylla* have also been identified as Al hyperaccumulators concentrating 11,000; 15,000; 10,000 and 3,000 mg Al kg^{-1} in dried leaves, respectively (Cuenca and Herrera, 1987; Ma et al., 1997; Shen et al., 2006; Zeng et al., 2011). The specific mechanisms of uptake and detoxification for Al are starting to be elucidated with the discovery of a protein in the Nramp family (natural resistance-associated macrophage protein), named Nrat1 (Nramp aluminum transporter 1), that specifically transports Al^{3+} across the plasma membrane in rice roots (Xia et al., 2010). The Nrat1 protein, unlike the other Nramp proteins in rice, does not transport Cd, Mn, Fe, or Al-citrate complexes, and contradictorily, the knockout of the Nrat1 protein increased Al sensitivity, but decreased Al uptake and increased Al binding to the cell wall (Xia et al., 2010). The increase in Al sensitivity is thought to be a result of an increase in apoplastic Al

damage from the increased binding to the cell wall (Barceló and Poschenrieder, 2002). The functional role of a plasma membrane transporter for a toxic metal (Al^{3+}) with no metabolic function is still unknown, however Huang et al. (2012), have characterized another protein in rice, OsALS1, a ABC transporter in the antigen processing subgroup that sequesters Al^{3+} into vacuoles internally detoxifying it.

For hyperaccumulators, the internal detoxification of Al has been studied, but not the transport mechanisms. In *H. macrophylla,* Negishi et al. (2012) identified two Al-transporters in the blue sepals in the Aquaporin family. In *H. macrophylla*, the flowers are blue when there is a high concentration of Al^{3+} in the vacuoles. The two transporters, HmPaLT1 (*H. macrophylla* plasma membrane aluminum transporter) and HmVaLT (*H. macrophylla* vascular aluminum transporter) move Al into the cytoplasm of the sepals and detoxify it via sequestration in vacuoles (Negishi et al., 2012). These proteins were not found in the rest of the plant, however transport of Al from the soil to the flowers is needed, and Negishi et al. (2012) have subsequently started studying the long distance transport of Al in *H. macrophylla*. However, Ma et al. (1997) found that Al is bound to citric acid within the cell sap via nuclear magnetic resonance and ion-exclusion chromatography, and this non-toxic form of Al may be a mechanism of transport. In accordance with a membrane protein transferring Al into the plant cells, Cuenca et al. (1991) measured an increased permeability of the endodermal cells to Al in the hyperaccumulator *R. grandis*, which was absent in the non-accumulator. Lastly, the influx of different Al species into *Camellia oleifera* was measured by Zeng et al. (2011) who found the Al^{3+} had the highest affinity for the roots while Al-citrate and Al-oxalate complexes had a low affinity for the roots cells, concurring with the observation that an Al^{3+} specific transporter may be present as it is in rice cells.

Comparisons between the specific soils and smelter-related metal uptake patterns can be explained by the available metal concentrations in the soil and the health of the plants. In both the non-limed soils, the concentrations of Cu, Zn and Cd in the roots was significantly ($P < 0.10$) higher than those plants grown in the reference soil—correlating with the higher concentrations of these metals in the soil. In the SVN soil, the root concentrations of Cu, Zn and Cd were 29×, 77× and 23× higher than the reference respectively, with Al concentrations 0.56× that of the reference. In the KN soil the root concentrations of Cu, Zn and Cd were 5.7×, 14× and 10× times higher than the reference, again with Al concentrations 0.80× those

plants grown in the reference soil despite the high concentration of available Al in the soil. For Al, the higher root concentrations in the healthy plants in the reference soil suggests an increased extraction of Al from the soil, as seen with other hyperaccumulators; e.g., Zn uptake by *T. caerulescens* (Lasat et al., 2000). The shoot concentrations in *P.* sp. 'Frensham' in the non-limed soils, however, did not follow a similar pattern. For the plants in the SVN soil, the shoot concentrations of the metals were highly variable, opposite to the excluders. Despite the obviously higher mean shoot content of Cu (9.1×) and Al (1.8×) to the shoots of the plants in the reference soil, only the Zn (13×) and Cd (9.5×) content in the shoots was statistically larger, likely due to varying levels of uptake with the poor survival of this plant on the SVN soil. In the KN soil where *P.* sp. 'Frensham' grew slightly better, the shoot concentrations of Cu (6.7×), Zn (3.1×) and Cd (3.2×) were significantly ($P < 0.10$) larger than the plants in the reference soil as expected, due to the better growth in this soil, which resulted in better metal uptake and less variable metal concentrations (Fig. 4.11). Shoot Al concentrations in the KN non-limed soil, on the other hand, were only 3.1× the concentration in plants grown in the reference soil despite the very different concentrations of available Al in these soils (i.e, 460 mg kg^{-1} in the KN non-limed soil and 78.3 mg kg^{-1} in the reference soil). These relatively higher shoot Al concentrations in the reference and limed soils reflect the high rate of Al accumulation in the shoots of *P.* sp. 'Frensham', which concentrated the metal in the aboveground foliage of the plants.

The dolostone applied to the soil by the *Green project* decreased the metal concentrations in *P.* sp. 'Frensham', but the reduction was not always statistically significant. In the SVN soil, liming statistically ($P < 0.10$) decreased the root concentrations to those plants in the non-limed soil for Cu, Zn, and Cd by 69%, 42%, and 41% respectively, while Al concentrations on the other hand increased in the roots by 42% with liming. For the shoots, liming did not statistically decrease any of the smelter-related metal concentrations in the plants compared to those in the non-limed soil, which averaged 27%, 56%, and 38% for Cu, Zn, and Cd, respectively. As with the roots, the shoot Al concentrations increased with liming in the SVN soil by 58%. Compared to the excluders *S. vulgaris* and *A. capillaris* in the SVN soil, *P.* sp. 'Frensham' was the only plant to increase shoot Al with liming, and have the lowest reduction in shoot Zn and Cd, displaying the differences in metal uptake between the metallophytes.

In the KN soil, liming significantly ($P < 0.10$) decreased the metal concentrations in the roots of the plants to those in the non-limed soil by 64% and 51% for Zn and Cd, respectively (Fig. 4.11). On the other hand, root concentrations of Cu and Al increased with liming by 9.3% and 140% respectively, despite the large difference in available Cu^{2+} and Al^{3+} of 101 mg kg^{-1} and 451 mg kg^{-1}, respectively, between the non-limed and limed soils. Contrary to the roots however, the shoot concentrations of Cu and Zn in the KN limed soil were statistically lower, decreasing by 71% and 64% respectively, but not Cd and Al which were still reduced by 56% and 46%, respectively. Despite the higher root concentrations of Cu and Al in limed soil and potential for bioconcentration, liming did not increase the shoot Cu or Al in the KN soil. Again, a number of soil and growth factors could have contributed to the lack of Cu and Al accumulation in the shoots, including interactions with other heavy metals or contaminants, growth dilution in the tissues of the large plants, soil adhering to the roots before digestion and analysis, or toxicity and high rates of uptake in the non-limed soil. Compared to the excluders however, in the SVN soil, *P*. sp. 'Frensham' still behaved differently having the lowest reduction in shoot Zn, Cd, and Al with liming compared to the other two plant species, due to an increase in shoot accumulation with the healthy plants.

Comparing the plants in the limed soils to the reference soils, like *S. vulgaris* and *A. capillaris,* the concentrations of Cu, Zn, and Cd in the roots were statistically higher when grown in the SVN and KN soils. In the SVN limed soil the root concentrations reached 8.7×, 45×, 12.9×, and 0.80× the concentration of the plants in the reference soil for Cu, Zn, Cd, and Al, respectively. In the KN limed site the root concentrations reached 22×, 5.2×, 5.0×, and 1.9× the concentration of the plants in the reference soil for Cu, Zn, Cd, and Al respectively. Where the relative available metal concentrations in the Flin Flon soil were higher, there was a greater increase in the root concentrations relative to plants in the reference soil. This pattern only differed for Al in the KN limed soil where the reference soil had 70.31 mg kg^{-1} more available Al^{3+}, but 48% less Al in the roots, which again may have been from soil contamination of the roots from the KN soil. For the shoot concentrations, Zn (12×) and Cd (9.4×) were statistically larger in the SVN limed soil compared to those plants grown in the reference soil, while the concentrations of Cu and Al only reached 3× those in the reference soil. While there was no significant difference in the shoot metals when the plants were grown in the KN limed soil, the shoots still averaged concentrations 1.9×, 0.97×, 1.4×, and

1.1× of those in the reference soil, with Zn decreasing. *Pelargonium* sp. 'Frensham' has been shown to accumulate Cu and Zn in the aboveground biomass, and hyperaccumulate Cd, which aids in explaining the differences in the shoot concentrations (Saxena and KrishnaRaj, 1999; Dan et al., 2000). Assuming the toxicity symptoms of the plants in the SVN non-limed soil suggest the normal mechanisms of metal uptake have broken down and the plants are experiencing an uncontrolled level of metal uptake, these plants should have the highest shoot metal concentrations, and they typically do, except for Cu. For the healthier plants, the shoot metal concentrations of Cu and Zn correlate with the available concentrations in the soil, as seen with wide ranging metal concentrations between populations (Zhao et al., 2003). For Cd however, despite the correlation between the available concentration in the soil and shoot metal concentrations, some accumulation of Cd in the shoots is still evident. Compared to the excluders (*S. vulgaris* and *A. capillaris*), the shoot Cd concentrations between the plants in the reference, SVN limed soil, KN non-limed soil, and KN limed soil are relatively even, indicating the healthy plants are accumulating Cd in the shoots as expected. For Al, there was no statistical difference between the root and shoot metal concentrations in *P.* sp. 'Frensham' regardless of the available concentrations in the soil and high shoot concentrations in the SVN limed soil, again indicating the high rate of uptake in the healthy plants is similar to the uncontrolled uptake in the dying plants. To date, accumulation of Al in this plant has not been documented, however given the differences in the available Al^{3+} concentrations between the five soils, the similarity in root and shoot concentrations is unexpected. In agreement with the correlation data, *P.* sp. 'Frensham' is showing characteristics signs of shoot-Al accumulation.

4.2.3.3 Low molecular weight organic acids (LMWOA). Organic acid profiles in the soils planted with *P*. sp. 'Frensham' (Fig. 4.12) differed from those in the soils planted with *S. vulgaris* and *A. capillaris* (Figs. 4.4 and 4.8, respectively). In general, LMWOA concentrations in soils planted with the hyperaccumulator were much lower than those in soils planted with either of the excluders. Moreover, LMWOA concentrations in the reference soil were generally comparable to those in the SVN and KN limed soils, though tartaric aid concentrations in the reference soil were somewhat greater ($P < 0.10$) than those in the SVN or KN soils.

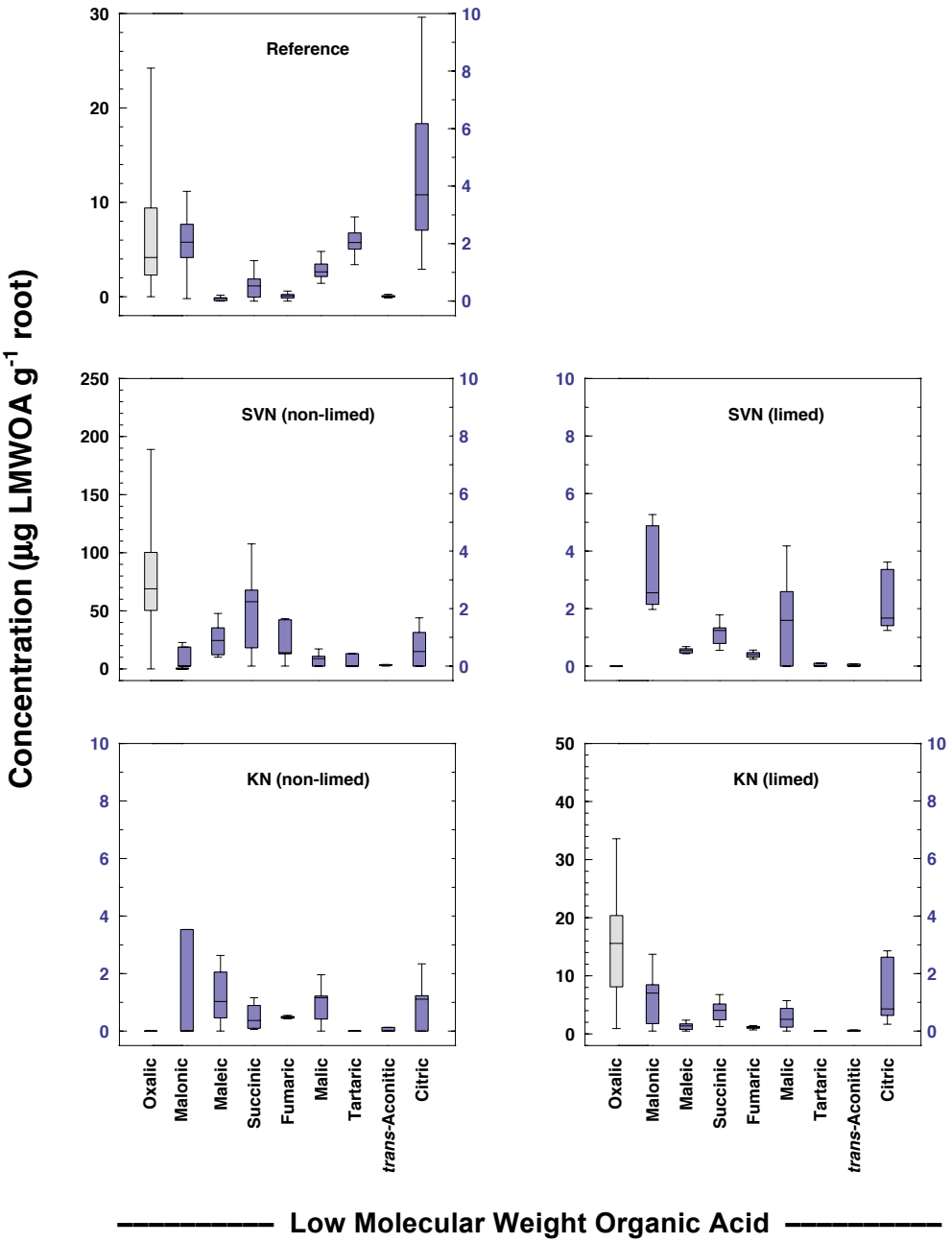

Fig. 4.12. Box and whisker plots of the low molecular weight organic acids (LMWOA) released into the soil by *Pelargonium* sp. 'Frensham' (n = 6) growing on the reference, Second Valley North (SVN) non-limed and limed, and Knight North (KN) non-limed and limed soils. Note: where present, the right-hand y-axis corresponds to the blue box & whisker bars. The data median (central tendency line), 1st and 3rd quartiles (bottom and top of box) and minimum and maximum values (bottom and top of whiskers) are displayed.

Low molecular weight organic acid concentrations in the SVN soil typically decreased in response to liming (Fig. 4.12)—particularly oxalic acid, which all but vanished in the limed soil. At the same time, concentrations of malonic and citric acids in the SVN soil tended to increase ($P < 0.10$) in response to liming. Liming the KN soil resulted in much greater concentrations of oxalic acid ($P < 0.10$), but otherwise had no significant effect on the LMWOA profile (Fig. 4.12).

Previous studies have identified malic and tartartic acids as the predominant LMWOAs in the tissues of *Pelargonium* species—with tartaric acid comprising as much as 1.5% of the plant dry weight (Ranjan and Rao, 1955; Williams and Harborne, 2002). In the present study, these LMWOAs were present at relatively low concentrations in the rhizosphere of the reference soil, relative to the non-limed SVN and KN soils, which suggests that they are being retained in the plant itself. On the other hand, greater concentrations of maleic acid in both the SVN and KN non-limed soils, and of fumaric acid in the SVN non-limed soil, may be a result of metal-induced stress. In fact, there were significant ($P < 0.01$) correlations between the available Cu^{2+}, Zn^{2+} and Cd^{2+} in the soil and the concentrations of both fumaric acid ($r = 0.608$, 0.578 and 0.563, respectively) and maleic acid ($r = 0.599$, 0.666 and 0.603, respectively) in the rhizosphere. On the other hand, tartaric acid was negatively correlated with available Cu^{2+} and Cd^{2+} ($r = -0.558$, $P < 0.01$ and $r = -0.469$, $P < 0.05$, respectively), but not with Zn^{2+}. Likewise, LMWOA concentrations in the soil were not correlated with shoot concentrations of either Al^{3+} or Zn^{2+}. Indeed, there appeared to be no significant relationship between metal uptake by *P.* sp. 'Frensham' and LMWOA concentration (individual or total) in the rhizosphere. Other studies that have analyzed how hyperaccumulator plants affected pH, dissolved organic carbon content, and available metal concentration in the rhizosphere also found that the hyperaccumulators extract metals from the soil without significantly altering the chemistry at the soil-root interface (Zhao et al., 2001; Puschenreiter et al., 2003; Dessureault-Rompre et al., 2008; Quartacci et al., 2009). Studies with *Thlaspi caerulescens* (a well characterized hyperaccumulator) also confirm this observation; e.g., Lasat et al. (2000) reported that high plant uptake of Zn by *T. caerulescens* was due to an increase in Zn transporters in the plant roots—and not mobilization of the metal in the soil. At present, little is known regarding the mechanism(s) involved in metal uptake by *P.* sp. 'Frensham'; however, it appears unlikely that plant production, and release into the rhizosphere, of

organic acids contributes to the high rates of uptake measured in this study. Instead, organic acids within the plant may be used to transport metals to the shoots of the plant, while some of the oxalic acid found in the reference, SVN non-limed, and KN limed soils may have inhibited the uptake of Al.

4.3 Comparison of Heavy Metal Uptake and Low Molecular Weight Organic Acids Production by Silene vulgaris, Agrostis capillaris, and Pelargonium sp. 'Frensham'

4.3.1 Root:shoot ratios of heavy metals between plants

The root-to-shoot ratio (r:s) of tissue metal concentration is a useful measurement for comparing metal excluding plants to metal hyperaccumulating plants as it indicates whether a metal is being sequestered in the roots (i.e., r:s > 1) or if the metal is being preferentially translocated to the shoots (i.e., r:s < 1) (Zhao et al., 2003; Yang et al., 2004). The r:s ratios of Cu, Zn, Cd, and Al in *S. vulgaris, A. capillaris,* and *P.* sp. 'Frensham' are illustrated in Table 4.2. In general, *A. capillaris* had the highest r:s ratios for all metals except Cu, confirming immobilization of metals in the roots of this species. Moreover, for each plant species, significant ($P < 0.10$) differences in the r:s values were related to the differences in the concentration of available metals in the soil, as well as to the health and survival of the plants. For example, healthy plants of *P.* sp. 'Frensham' yielded the lowest r:s ratio for all metals (indicating enhanced transport of the heavy metals to the shoots), but especially for Al with several individual plants having r:s values < 1 (indicative of hyperaccumulation). Where the available metal concentration was very low, or where severe metal stress/plant death occurred, r:s values approached 1.0, reflecting either reduced metal uptake or the uncontrolled uptake of metals into the shoots, respectively—masking differences between the excluders and hyperaccumulator. Such was the case with Zn, which exhibited r:s ratios < 1 for plants grown in the reference soil (Table 4.2); indeed, Zn r:s values for *S. vulgaris*, *A. capillaris*, and *P.* sp. 'Frensham' were 0.77, 0.98 and 0.38, respectively. In addition, differences in the Zn r:s values for *P.* sp. 'Frensham' and the excluder species in the reference were significant ($P < 0.10$), signifying enhanced translocation of Zn into the shoots of *P.* sp. 'Frensham'. The average concentration of Zn in the shoots of plants grown in the reference soil also was greatest for *P.* sp. 'Frensham' (110 mg kg^{-1}) and was 32% greater

than that in *S. vulgaris* (84 mg kg^{-1}) and 96% greater than that in *A. capillaris* (56 mg kg^{-1}). In the case of *P.* sp. 'Frensham', this is somewhat greater than the upper range for terrestrial plants reported by Shaw et al. (2004) (i.e., mean Zn concentration ranging from 8 to 100 mg kg^{-1}). The only other r:s ratios ≤ 1 were observed for Zn in the *S. vulgaris* grown in the SVN non-limed (0.95) SVN limed (0.97), and KN non-limed (1.0) soils. However, given the very high concentrations of available Zn^{2+} in the SVN limed soils with the death of the plants in both the non-limed soils—it is suggested that the high uptake of Zn into the shoots (i.e., 27,000 mg kg^{-1} and 930 mg kg^{-1}, and 3,300 mg kg^{-1}, in the SVN non-limed, SVN limed, and KN non-limed soils, respectively) represents an uncontrolled accumulation.

Table 4.2. Root:shoot ratios of Cu, Zn, Cd, and Al concentrations for *Silene vulgaris* (n = 6), *Agrostis capillaris* (n = 6), and *Pelargonium* sp. 'Frensham' (n = 6) grown in the reference, Second Valley North (SVN) non-limed, SVN limed, Knight North (KN) non-limed, and KN limed soils.

Soil (± lime)	Plant species	Root-to-shoot (r:s) ratio			
		Cu	Zn	Cd	Al
Reference	*S. vulgaris*	3.1 a	0.77 a	2.6 a	28 a
	A. capillaris	1.3 b	0.98 a	14 a	63 a
	P. sp. 'Frensham'	1.7 b	0.38 b	19 a	5.5 b
SVN (- lime)	*S. vulgaris*	8.3 b	0.95 a	2.4 b	7.2 b
	A. capillaris	20 a	1.9 a	12 a	81 a
	P. sp. 'Frensham'	5.8 b	1.9 a	3.3 b	2.1 b
SVN (+ lime)	*S. vulgaris*	23 a	0.98 b	1.1 c	19 a
	A. capillaris	15 a	4.6 a	15 a	82 a
	P. sp. 'Frensham'	4.8 a	1.5 b	3.8 b	2.2 b
KN (- lime)	*S. vulgaris*	3.1 b	1.0 b	4.0 b	8.8 a
	A. capillaris	12 a	4.5 a	22 a	49 a
	P. sp. 'Frensham'	4.0 b	1.3 b	4.2 b	1.7 a
KN (+ lime)	*S. vulgaris*	100 a	6.1 a	5.2 a	89 a
	A. capillaris	43 b	10 a	81 a	110 a
	P. sp. 'Frensham'	16 b	1.6 b	5.3 a	11 b

† Within soil type groups, means followed the same letter are not significantly different ($P < 0.10$); significance was determined using the Kruskal-Wallis and Games-Howell tests.

Overall the r:s ratios exhibited similar patterns in the reference and KN limed soils where all three plant species were healthy and grew vigorously; and in the non-limed soils were all three species exhibited severe signs of metal toxicity and/or death (Table 4.2). In the reference and KN limed soil, *P*. sp. 'Frensham' had the lowest r:s ratios for Zn and Al, while differences in the r:s ratios for *S. vulgaris* and *A. capillaris* were not significant. On the other hand, in both of these soils *S. vulgaris* had the highest r:s ratio for Cu, which is not surprising given the plants known tolerance to Cu (Song et al., 2004). For Cd, none of the r:s ratios were statistically different in the reference and KN limed soils, even though the r:s ratio for *A. capillaris* was roughly 15-times higher than that of either *S. vulgaris* or *P*. sp. 'Frensham'—reflecting the high variability in the data.

In the non-limed soils, *A. capillaris* always had the highest r:s ratios for the metals, reflecting the adaptation of this species to the smelter-impacted soils—especially at the SVN site—and high rate of metal sequestration in the roots. Conversely, the r:s ratios in *S. vulgaris* and *P*. sp. 'Frensham' were statistically similar for all the metals in the non-limed soils, despite differences in the metal tolerance strategies employed by the two plants. However, given the fact that *S. vulgaris* died shortly after being transplanted into the non-limed soils, while *P*. sp. 'Frensham' survived (see Figs. 4.1 and 4.9), the lower r:s ratios for *S. vulgaris* are most likely due to the uncontrolled uptake of metals into the shoots of the plant—mimicking the high rate of metal translocation seen with the hyperaccumulator. The only exceptions to this pattern on the non-limed soils occurred where there were exceedingly high concentrations of available metals in the soil (i.e., Zn on the SVN soil and Al on the KN soil). For both of these metals there was no significant difference in the r:s ratios between the three plant species; presumably due to high rates of shoot metal uptake in the metal-stressed plants in these soils.

Lastly, the plants grown in the SVN limed soil yielded r:s ratios for the three plant species that were different from those in the other soils. These differences reflect the varying effects of the metal concentrations in the soil, the toxicity experienced by *S. vulgaris*, the vigorous growth of *A. capillaris*, and the survival of *P*. sp. 'Frensham'. In the absence of excessively high concentrations of available Cu^{2+} in the soil, differences between species in the r:s ratios of Cu were not significant, suggesting Cu uptake was tightly controlled. For Zn and Cd, *A. capillaris* had the highest r:s ratio due to root sequestration of the metals; while *S.*

vulgaris and *P*. sp. 'Frensham' had similar ratios reflecting the higher rates of translocation in the geranium and the metal-induced stress suffered by *S. vulgaris*. Finally, *P*. sp. 'Frensham' had a significantly lower r:s ratio for Al in the SVN limed soil, with shoot metal concentrations reaching levels equivalent to hyperaccumulation.

The specific r:s ratios for the metals were generally in agreement with those of metal tolerant plants reported in other studies. However, most, if not all, metal tolerance and uptake studies for excluders focus on tolerance indices of root growth and germination, as well as shoot metal concentrations. The focus for hyperaccumulators (most of which have been studied in hydroponic systems), on the other hand, is shoot metal concentration. Specific root and shoot concentrations of Cu, Zn, Cd, and Al for the plant species used in the present study have not been reported; however, r:s ratio can be calculated from the few studies reporting root and shoot metal concentrations for other plants. For *A. capillaris*, Dahmani-Muller et al., (2000) measured the Cu, Zn, and Cd concentrations in plants surrounding a non-ferrous metallurgical site in Northern France. Based on the reported root and shoot concentrations, the average r:s ratio for Cu, Zn, and Cd was 3.3, 1.7 and 3.3, respectively; indicating that the metals were being immobilized in the roots. These r:s ratios were within the range or, in the case of Cd, smaller than the values reported in my study (see Table 4.2). The differences in the r:s ratios could be due to differing available concentrations in the soil, for which the values were not reported by Dahmani-Muller et al. (2000). For *S. vulgaris*, the r:s ratios ranged from 3.1 to 103, 0.77 to 6.1, 1.1 to 5.2, and 7.2 to 89 for Cu, Zn, Cd, and Al respectively, in which the range between the values was generally higher for each of the metals compared to *A. capillaris*, reflecting the greater range in death and survival of the plants during the trial.

On the other hand, for the Zn/Cd hyperaccumulator *Sedum alfredii*, the r:s ratio of Cd was always less than 0.5 when grown in hydroponic solutions with Cd concentrations ranging from 12.5–200 μmol L^{-1}; and below 0.3 for Zn in hydroponic solutions ranging from 1–500 μmol L^{-1} (Yang et al., 2004; Yang et al., 2006). In the present study, the r:s ratios for Cu, Zn, Cd, and Al in *P*. sp. 'Frensham' ranged from 1.7 to 16, 0.38 to 1.9, 3.3 to 19, and 1.7 to 11, respectively. For Cu, Zn, and Al the average r:s ratios were not <1, which was a function of either the low availability of metals in the limed soils, or root damage/metal stress in the non-limed soils. However, it is difficult to directly compare the results from a

hydroponic study to a potted study due to the differences in metal availability between the two media, as well as the multiple factors interacting in the soils with plant growth including other toxic metals, pH and nutrient availability. Compared to the excluders, the average range of r:s values for *P*. sp. 'Frensham' was much lower, indicative of the differences in metal tolerance strategies.

From the literature it is clear the r:s ratios are generally >1 for metal excluding plants, and <1 for metal hyperaccumulators—at least when compounding variables are controlled, as in hydroponics experiments. In the present study the r:s ratios for Cu, Zn, Cd, and Al were not as clearly delineated between the excluders and hyperaccumulator due to the varying concentrations of available metals in the soils, differences in the growth and survival of the plants, and possible interactions between contaminants in the soils. However, the consistently higher r:s ratios for *S. vulgaris* and *A. capillaris*, as well as the lower r:s ratios for *P*. sp. 'Frensham', concur with the known mechanisms of metal tolerance reported for these plants. Moreover, patterns in the r:s ratios between the plants could be explained and possibly predicted by understanding the metal uptake strategies of the plants, the metal concentrations in the soil, and plant growth and survival.

4.3.2 Low molecular weight organic acids in root-affected soil

Low molecular weight organic acids measured in the soil differed between the three plant species with respect to the total concentrations and the presence of specific acids (Fig. 4.13). The reference soil planted with *S. vulgaris* or *A. capillaris* had significantly ($P < 0.10$) more total LMWOAs than the soil planted with *P*. sp. 'Frensham,' indicating that these plants have naturally higher concentrations of LMWOAs in the root-affected soil when healthy. In terms of specific LMWOAs in the reference soil, however, only *A. capillaris* had a statistically higher concentration of citric acid compared to *P*. sp. 'Frensham,' while *P*. sp. 'Frensham' yielded higher concentrations of maleic, succinic, and tartaric acid than *A. capillaris*—and all of which are found in naturally high concentrations in the plant tissues (Ranjan and Rao, 1955; Williams and Harborne, 2002). The specific LMWOAs found in reference soils planted with all three species were similar, though oxalic and malonic acid concentrations were consistently higher in the *S. vulgaris* system.

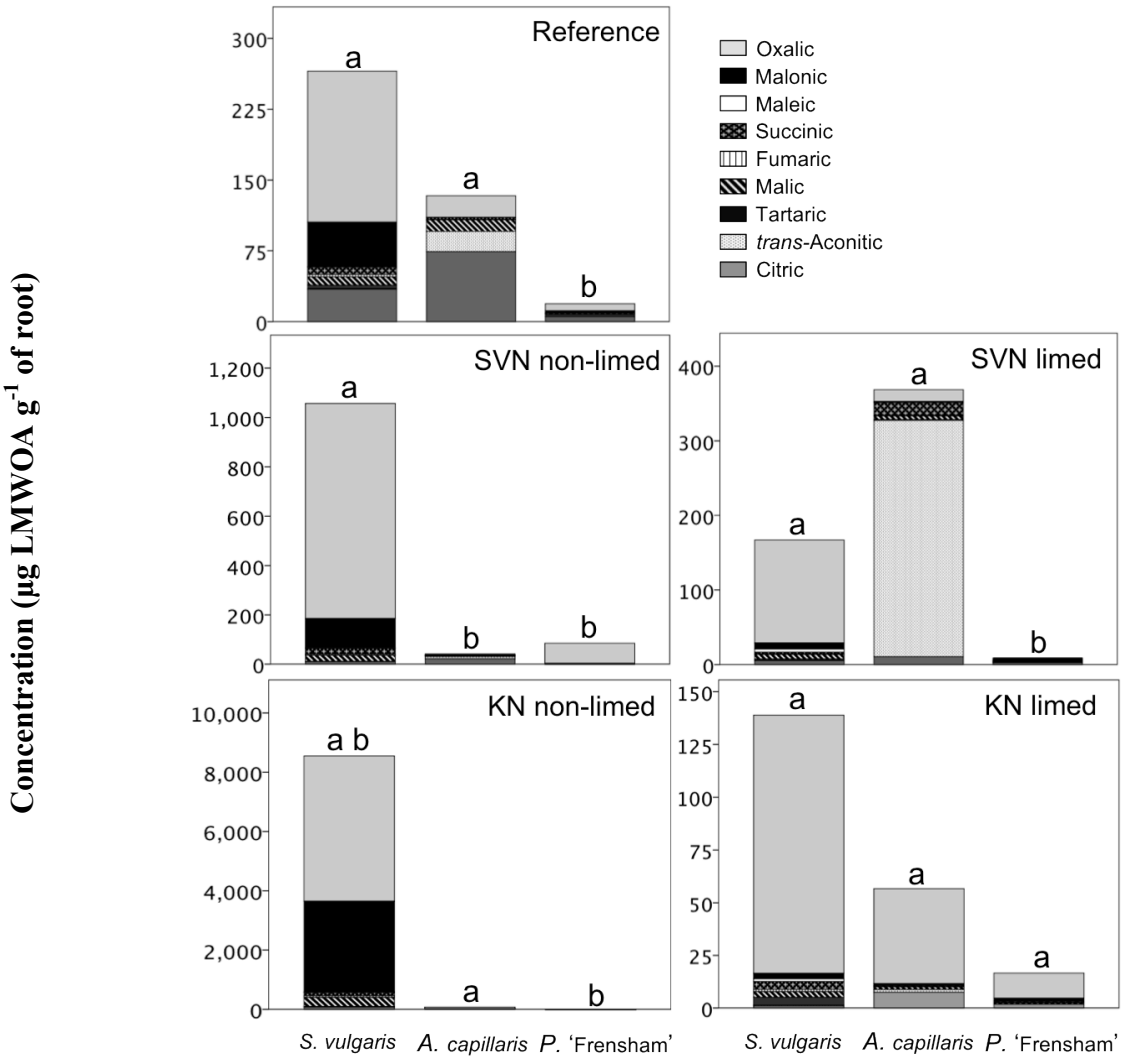

Fig. 4.13. Low molecular weight organic acids (LMWOA) in the soils for *Silene vulgaris* (n = 6), *Agrostis capillaris* (n = 6), and *P.* sp. 'Frensham' (n = 6) when grown on the reference soil, SVN non-limed soil, SVN limed soil, KN non-limed soil, and KN limed soil. Letters above the bar represent significant (P < 0.10) differences in the total organic acid content in the soil determined by the Kruskal-Wallis and Games-Howell tests. Note the scale differences on the y-axes between the soils.

In the non-limed soils, the concentration of total LMWOAs and oxalic acid was drastically higher for *S. vulgaris* than for either *A. capillaris* or *P.* sp. 'Frensham', but the differences were statistically significant only in the SVN non-limed soil, which also exhibited the highest rates of plant survival. Only fumaric acid was larger for *S. vulgaris* compared to *A. capillaris* in the KN non-limed soil. Between *A. capillaris* and *P.* sp. 'Frensham', these plants had similar total LMWOAs in the SVN-non-limed soil where *A.*

capillaris was the only plant to exhibit growth at a minimum of 80% of the reference plants, inferring the lower metal stress decreased the LMWOAs in the soil to comparable levels of *P*. sp. 'Frensham.' Where the metal stress increased on *A. capillaris*, as in the KN non-limed soil, the total LMWOA and succinic acid were statistically higher than *P*. sp. 'Frensham'.

In the SVN limed soils, *S. vulgaris* and *A. capillaris* had significantly higher concentrations of total LMWOAs compared to *P*. sp. 'Frensham', where signs of metal stress were still prevalent (despite the liming). *Silene vulgaris* also had the highest concentrations of the oxalic acid in this soil while *A. capillaris* had the highest concentrations of *trans*-aconitic acid. For the SVN limed soil, *P*. sp. 'Frensham' had more maleic acid in the root-affected soil compared to *A. capillaris* only. All of the relationships between specific LMWOA in the soils can be related back the concentration of these acids within the plant species.

On the other hand, in the KN limed soil where none of the plants displayed any signs of metal stress or toxicity during the growth trial, there were no significant differences between the total LMWOAs for any of the plants species—likely due to a decrease in the LMWOAs released by the excluders. For specific LMWOA, *S. vulgaris* still had significantly more maleic acid than *A. capillaris* and more malic acid than *P*. sp. 'Frensham.'

The high LMWOA concentrations found in the root-affected soil for the two excluders, *S. vulgaris* and *A. capillaris*, is a function of metal stress and the naturally higher concentration of LMWOAs released to the soil evident from the reference pots. Metal tolerant excluders have been found to increase metal dissolution in the rhizosphere, increase or decrease the pH, and increase the dissolved organic carbon content around the roots under metal stress (Puschenreiter et al., 2003; Dessureault-Rompre et al., 2008; Quartacci, et al., 2009). For most of the metals studied in previous works, the strongest correlations exist between Al toxicity and LMWOA exudation (Delhaize et al., 1993; Zheng et al., 1998; Lasat et al., 2000; Mariano and Keltjens, 2003; Sasaki et al., 2004). For this trial, the soils where the available Al^{3+} was high and the excluders survived transplantation, the total LMWOAs in the soils were higher than the hyperaccumulator likely die to the exclusion of Al from root uptake. Specific exclusion of metals other than Al has been harder to definitively prove given the metabolic need, or mimicked behavior of micronutrients, for Cu and Zn, and Cd (Prasad, 2004). For the hyperaccumulator *P*. sp. 'Frensham,' the overall lower total LMWOAs in the

soils from the growth trial concurs with the observation that active foraging for heavy metals linked with an efficient system of transporters on the root cell is more important than increasing the metal solubility in the soil for uptake (Lasat et al., 2000; Zhao et al., 2001).

4.3.3 Extraction of metals from the soil, changes in pH, and changes in available metal concentrations

The extraction of metals from the soil was assessed by determining the percentage of the available metals removed by the roots and shoots of each plant (Table 4.3) based on: (i) the concentration of available metals in the soil, (ii) the dry weight of the soil in each pot, (iii) the concentration of each metal in the roots and shoots of the plant, and (iv) the dry weight of the roots and shoots. In general, extraction efficiencies for metals were better where the concentrations were low to moderate for all three plants. For *S. vulgaris*, the percentage of metals in the roots ranged from 0.10% to > 100% of the available metals in the soil, with the largest values occurring for Al in the KN limed soil. For the shoots, the percentage of metals never exceeded 15% of the available metals in the soil, with the largest values being 12% for Zn in reference soil and 11% for Al in the KN limed soil. The low accumulation of metals in the aboveground biomass is due to the exclusion of metals from the shoots of healthy plants and, in the case of *S. vulgaris*, to the low biomass production. The extraction of available metals from the soils was accompanied by a significant increase in pH in the SVN non-limed soil (0.11 units) and SVN limed soil (0.27 units) compared to the unplanted control (Table 4.3). With the change in pH in these soils, growth of *S. vulgaris* significantly ($P < 0.10$) decreased the concentration of available Al^{3+} in the SVN non-limed soil compared to the unplanted control (i.e., by 21%), and available Cu^{2+} and Al^{3+} (i.e., by 40% and 56%, respectively) (Table 4.4).

For *A. capillaris*, the percentage of available metals removed from the soil to the roots ranged from 0.91% for Cu in the KN non-limed soil to > 100% for Al in the KN limed soil. The shoot metal extraction ranged from 0.08% to 39.8%; with the highest values for Cu in the reference soil. Like *S. vulgaris*, the growth of *A. capillaris* significantly increased the pH in the SVN non-limed soil (0.34 units) and SVN limed soil (0.44 units) compared to the unplanted control. However, unlike *S. vulgaris*, significant changes in the available metal concentrations in the soil were not limited to the SVN site—although *A. capillaris*

Table 4.3. Percentage of the total available metals removed by the roots and shoots of *Silene vulgaris* (n = 6), *Agrostis capillaris* (n = 6), and *Pelargonium* sp. 'Frensham' (n = 6) for whole pots when grown in the reference soil, Second Valley North (SVN) non-limed soil, SVN limed soil, Knight North (KN) non-limed soil, and KN limed soil.†‡

Soil (± Lime)	Plant Species	Cu		Zn		Cd§		Al	
		Roots	Shoots	Roots	Shoots	Roots	Shoots	Roots	Shoots
		%							
Reference									
	S. vulgaris	5.90 b	1.77b	14.7 a	11.9 a	-	-	67.4 a	2.16 b
	A. capillaris	39.8 ab	27.5 ab	19.9 a	16.3 a	-	-	> 100 a	3.85 b
	P. sp. 'Frensham'	39.5 a	25.2 a	41.4 a	129 b	-	-	81.9 a	13.1 a
SVN (- lime)									
	S. vulgaris	1.08 c	0.15b	0.17 b	0.27 b	0.15 c	0.10 b	1.19 b	0.60 b
	A. capillaris	8.65 a	0.47ab	2.41 a	1.41 a	2.62 a	0.21 a	> 100 a	2.34 a
	P. sp. 'Frensham'	2.52 b	0.77a	1.83 a	1.23 a	1.09 b	0.45 a	8.19 b	3.62 a
SVN (+ lime)									
	S. vulgaris	8.24 a	0.40b	0.41 b	0.46 c	0.56 b	0.56 ab	51.3 b	2.86 b
	A. capillaris	46.0 a	3.01a	3.71 a	0.82 b	6.16 a	0.29 b	> 100 a	6.63 a
	P. sp. 'Frensham'	13.9 a	3.84ab	4.58 a	3.21 a	3.49 a	1.03 a	45.2 b	45.1 ab

† Letters represent significant differences ($P < 0.10$) based on the Kruskal-Wallis and Games Howell tests.

‡ Low available metal concentrations in the soil along and/or soil contamination on the roots may produce percent values greater than 100.

§ Concentrations of available Cd in the reference soil were less than the detection limits of the MP-AES for most of the measurements; therefore, significant differences in the root and shoot extraction of available Cd could not be calculated.

Table 4.3 continued. Percentage of the total available metals removed by the roots and shoots of *Silene vulgaris* (n = 6), *Agrostis capillaris* (n = 6), and *Pelargonium* sp. 'Frensham' (n = 6) for whole pots when grown in the reference soil, Second Valley North (SVN) non-limed soil, SVN limed soil, Knight North (KN) non-limed soil, and KN limed soil.†‡

Soil (± Lime)	Plant Species	Cu		Zn		Cd§		Al	
		Roots	Shoots	Roots	Shoots	Roots	Shoots	Roots	Shoots
		%							
KN (- lime)									
	S. vulgaris	0.10 b	0.03 c	0.12 c	0.10 c	0.12 a	0.14 b	0.37b	0.07 b
	A. capillaris	0.91 a	0.08 b	1.28 b	0.31 b	2.40 b	0.15 b	3.56a	0.08 ab
	P. sp. 'Frensham'	1.55 a	0.43 a	2.87 a	2.34 a	2.77 b	0.82 a	1.14b	0.63 a
KN (+ lime)									
	S. vulgaris	38.6 b	0.42 c	41.5 a	9.14 a	12.2 a	2.94 ab	> 100 b	11.4 b
	A. capillaris	> 100 ab	2.89 b	62.8 a	5.63 a	14.2 a	0.24 b	> 100 ab	19.5 b
	P. sp. 'Frensham'	> 100 a	13.4 a	59.1 a	57.3 a	14.7 a	3.05 a	> 100 a	255 a

† Letters represent significant differences ($P < 0.10$) based on the Kruskal-Wallis and Games Howell tests.

‡ Low available metal concentrations in the soil along and/or soil contamination on the roots may produce percent values greater than 100.

§ Concentrations of available Cd in the reference soil were less than the detection limits of the MP-AES for most of the measurements; therefore, significant differences in the root and shoot extraction of available Cd could not be calculated.

Table 4.4. Concentration of 0.01*M* $CaCl_2$-extractable heavy metals in the reference, SVN non-limed, SVN limed, KN non-limed, and KN limed soils after the growth of *Silene vulgaris* (n = 6), *Agrostis capillaris* (n = 6), and *P.* sp. 'Frensham' (n = 6) compared to the unplanted control.

Soil (± lime)	Plant Species	Cu	Zn	Cd	Al	pH
		mg kg $^{-1}$				
Reference	*S. vulgaris*	0.33	0.69	UD	14.3	4.85
	A. capillaris	0.20	0.58	UD	9.70**	4.89
	P. sp. 'Frensham'	0.49	0.87	UD	16.9	4.77
	Unplanted control	0.31	0.68	UD	17.1	4.84
SVN (- lime)	*S. vulgaris*	21.5	201	2.25	26.7¶	4.32¶
	A. capillaris	17.5**	185*	2.09	15.4**	4.55***
	P. sp. 'Frensham'	20.0*	191¶	2.19	26.4¶	4.30
	Unplanted control	26.1	215	2.16	33.7	4.21
SVN (+ lime)	*S. vulgaris*	1.62**	77.8	0.77	5.29*	4.93**
	A. capillaris	1.78*	62.3**	0.70	5.54*	5.10**
	P. sp. 'Frensham'	2.32	76.6	0.73	8.73	4.82
	Unplanted control	2.70	87.1	0.73	12.1	4.66
KN (- lime)	*S. vulgaris*	38.3	40.7	0.66	271	3.76
	A. capillaris	42.9	42.6	0.70	253	3.77
	P. sp. 'Frensham'	35.5	32.6¶	0.61	252	3.80
	Unplanted control	42.7	43.1	0.67	288	3.69
KN (+ lime)	*S. vulgaris*	4.82	10.9	0.20	1.32	5.63
	A. capillaris	1.44*	2.51	0.25	1.42	5.23
	P. sp. 'Frensham'	1.12	0.74	0.20	0.90	5.67
	Unplanted control	0.97	2.12	0.22	0.91	5.46

† Significant values are based on the Kruskal-Wallis and Games Howell test compared to the unplanted control; ¶, *, **, and *** are significant at the 0.10, 0.05, 0.01, and 0.001 probability levels

significantly ($P < 0.10$) decreased the available concentrations of Cu^{2+}, Zn^{2+}, and Al^{3+} in the SVN non-limed by 33%, 14%, and 54%, respectively, and Cu^{2+}, Zn^{2+}, and Al^{3+} in the SVN limed soil by 34%, 28%, and 54%, respectively. In the reference soil, only the growth of *A.*

capillaris significantly decreased the available Al^{3+} (43%) compared to the unplanted control, despite the lack of a significant change in pH. *Agrostis capillaris* did remove over 100% of the available Al^{3+} to the roots of the plant; however, this fraction was not statistically different from *S. vulgaris* and *P*. sp. 'Frensham,' and was likely a combination of root uptake and soil adhering to the roots before digestion. In the KN limed soil, *A. capillaris* increased the available Cu^{2+} in the soil compared to the unplanted control by 48%, displaying the variable interactions between roots and soils under different conditions of metal stress and pH. For the KN limed soil, given the higher pH and the ability of *A. capillaris* to thrive on acidic soils, this increase in available Cu^{2+} may be due to nutrient acquisition strategies. Although the pH was not significantly different from the unplanted reference in the KN limed soil, *A. capillaris* generally decreased the pH of this soil—and a longer growth trial might display larger differences in pH relating to changes in available Cu^{2+}.

The extraction of available metals from the soils has not been sufficiently studied for excluders; however, studies have looked at changes in the rhizosphere and root-affected soils. For *A. capillaris*, Römkens et al. (1999) measured the changes in pH, soluble Cu^{2+}, and dissolved DOC in Cu contaminated soil after plant growth. The study found the growth of *A. capillaris* increased the pH, decreased the available Cu^{2+}, and increased the DOC in the planted pots, similar to the results from this study where the pH was below 5. Römkens et al., (1999) subsequently concluded *A. capillaris* was beneficial for immobilization and detoxification of Cu in the soil.

Lastly, for *P*. sp. 'Frensham' the percentage of the available metals removed from the soil was more variable, with root fractions ranging from 1.09% of the available Cd^{2+} in the SVN non-limed soil to >100% of the available Al^{3+} in the KN limed soil. However, like the other root measurements, contamination of adhering soil has likely increased the root fraction of metals (Table 4.3). For the roots, the extraction of available metals for *P*. sp. 'Frensham' was the lowest for Cu in the KN non-limed soil at 0.43%, which was still significantly ($P < 0.10$) higher than *S. vulgaris* and *A. capillaris*. The highest percentage of available metals removed to the shoots of *P*. sp. 'Frensham' was at 129% for Zn in the reference soil and 255% for Al in the KN limed soil. Consequently, *P*. sp. 'Frensham' was the only plant species in which metal uptake exceeded the available (i.e., 0.01*M* $CaCl_2$ extractable) soil metal pool (Table 4.3). Despite the large metal content in the roots and shoots of *P*. sp.

'Frensham,' no significant difference was measured in the pH of any of the soils after plant growth, concurring with theory that active foraging and efficient transporters in the roots of hyperaccumulators can account for the high metal uptake (Lasat et a., 2000) (Table 4.4). Like *A. capillaris* the growth of *P*. sp. 'Frensham' significantly ($P < 0.10$) decreased the available concentration of Cu^{2+}, Zn^{2+}, and Al^{3+} in the SVN non-limed soil by 23%, 11%, and 22%, respectively, however with no change in the pH of the soil, this decrease in available metals is due to root and shoot uptake, or another reaction in the soil immobilizing metals (Table 4.4). Moreover, *P*. sp. 'Frensham' was the only plant to significantly change the available metal concentrations in the KN non-limed soil, decreasing the available Zn^{2+} compared to the unplanted control by 65%. For the KN non-limed soil, *P*. sp. 'Frensham' removed significantly more of the available Zn^{2+} in the roots and shoots compared to both *S. vulgaris* and *A. capillaris,* which was related to the better growth of *P*. sp. 'Frensham' in this soil (Table 4.3).

For *P*. sp. 'Frensham,' again changes in the soil chemistry have not been strongly correlated with metal uptake and tolerance for hyperaccumulators, however decreases in the labile fraction of soil metals have (Lasat et al., 2000; Zhao et al., 2000). In a pot experiment with contaminated soil from a smelter affected site in Arnoldstein, Austria, Puschenreiter et al. (2003) separated the rhizosphere soil from the bulk soil with nylon bags. In Puschenreiter's et al. (2003) study, the hyperaccumulator *T. caerulescens* significantly decreased the labile fraction of Zn^{2+} in the rhizosphere. However, the reduction in labile Zn^{2+} could not account for the high concentration of Zn in the shoots of the plant, as with Zn in the reference soil and Al in the KN limed soil in this study. Pushenreiter et al., (2003) subsequently concluded that the depleted available fraction of Zn was re-supplied from the non-labile fraction of Zn in the soil. Similarly, where *P*. sp. 'Frensham' removed over 100% of the available Zn^{2+} from the reference soil and Al^{3+} from the KN limed soil, there were no significant changes in the labile fraction of these metals measured. However, this study used whole soils from the pots to represent landscape-scale changes applicable to Flin Flon, MB/ Creighton, SK, and not just the rhizosphere. Therefore, similar changes in the available metal fraction in the soil may or may not have been found in the rhizosphere-specific soils for *P*. sp. 'Frensham.'

5 CONCLUSION

For large, variable sites such as the HBMS site in Flin Flon/Creighton, predicting the response of soil amendments in relation to plant growth is difficult, given the wide-ranging metal concentrations and soil characteristics. This variability is evident even within the two smaller sites chosen for this study. For example, the SVN site was characterized by a coarser texture, a pH of 4.01 (similar to the reference soil), low carbonates, TOC, S, exchangeable bases, high concentrations of available Zn^{2+}, Cd^{2+}, and $Pb^{2+/4+}$, and, most importantly, low NO_3^-. On the other hand, soils at the KN site had a finer texture, a pH of 3.36, more NO_3^- and NH_4^+, low carbonates, high TOC, high S, low exchangeable bases, and high concentrations of available Cu^{2+} and Al^{3+}. Before liming, both of these soils were problematic for plant growth, but for different reasons related to the available metal concentrations and nutrient status. The effectiveness of the liming in relation to plant growth was based on natural revegetation of the sites ten years following the addition of dolostone. However, given the lack of biodiversity and a seed source, this can be misleading as greenhouse studies demonstrated that *A. capillaris* was capable of vigorous growth in the non-responsive (SVN) non-limed soil when fertilized. Nevertheless, there were differences between the effects the dolostone had on the soils related to the dissolution of the liming material on site and nutrient status of the soil related to Objective 1. At the SVN site, where the dolostone was found to be ineffective in terms of increasing the soil pH and promoting establishment of new vegetation, the lack of exchangeable Ca^{2+} and Mg^{2+} in the soil indicated that the dolostone either had not dissolved or that the Ca^{2+} and Mg^{2+} were leaching out of the coarse textured soil. This lack of dissolution, high rate of leaching, and poor diversity were likely due to the dense rhizomes and sod mat of the *A. capillaris* on site, inhibiting the direct contact of the lime with the soil and the establishment of other plant species. Moreover, the low nutrient status of this site compared to the responsive site (KN) was inhibitive of plant growth in the field.

For the three metal tolerant plants studied, plant growth in the soils was related to each plant's threshold of metal tolerance, exhibited by the reduction in biomass in the non-limed soils, except for *A. capillaris* which is native to the SVN site. Moreover, despite the differing mechanisms of metal tolerance, the growth and metal uptake patterns were generally similar

between the plants. The soils used in this study represented extremes from the HBMS site and the large differences in available metal concentrations and toxicity between the limed and non-limed soils made comparisons between the excluders and hyperaccumulator difficult. That is, the non-limed soils usually induced early plant death and the unregulated uptake of metals into the shoots of the excluder plants (i.e., *S. vulgaris* and *A. capillaris*). The only exception for growth was again *A. capillaris* in the SVN non-limed soil, in which this plant had the pre-determined genetic ability to tolerate the conditions in this soil due to the natural selection of the plant surviving at the field site in Flin Flon. Generally, the most noticeable difference between the plants was with the LMWOA, where the excluders contained more of these compounds in the contaminated soil than the hyperaccumulator. *Silene vulgaris* had more oxalic acid in the SVN non-limed soil compared to the reference and limed soils; *A. capillaris* had more maleic acid and succinic acid on the KN non-limed soil compared to the reference soil, and overall more *trans*-aconitic acid on the SVN-limed soil; while *P.* sp. 'Frensham' had more tartaric acid in the non-contaminated reference soil. However, the organic acids found in the soil were usually related to the concentration of these acids within the plant tissues themselves. And, with the severe toxicity of the non-limed soils, especially with *S. vulgaris* and *P.* sp. 'Frensham,' it was difficult to correlate the LMWOA in the soil with metal tolerance over cell lyses in the roots. Moreover, due to restrictions in sampling design, the "rhizosphere" soil itself could not be sampled, which may have resulted in lower concentrations of root-derived LMWOA and, in turn, masked differences between the three plant species.

Overall, the use of lime as a soil amendment was successful in alleviating metal toxicity in terms of plant growth regardless of the metal contamination; however, the direction and extent of these changes was related to dissolution of the dolostone and the inherent nutrient status of the soil. Many disturbed areas containing elevated concentrations of heavy metals from natural occurrences or anthropogenic activities develop a unique population of metal-tolerant flora adapted to the specific physical and chemical conditions present in the soil. In Flin Flon, this metal-tolerant population included *A. capillaris* at the SVN site. The invasive nature of *A. capillaris*, dense sod mat, and surface rhizomes has inhibited the establishment of other plant species, which has resulted in the *Green Project* classifying the soil as "nonresponsive" to liming. Without removing *A. capillaris*, it is

difficult effectively judge the responsiveness of the soil to liming, especially with respect to the growth of understory species. Nevertheless, in the field the freely available Zn^{2+} present in this soil, combined with its low nutrient status may still be problematic for the establishment of Boreal plant species in the future.

For Objectives 2a and 2b, the metal uptake patterns were similar between the excluders *S. vulgaris* and *A. capillaris* and the hyperaccumulator *P*. sp. 'Frensham,' despite the different mechanisms of metal tolerance and known partitioning of metals within the plants. An exception was noted for Al, which was shown to strongly accumulate in the shoots of *P*. sp. 'Frensham'. For *P*. sp. 'Frensham' in the SVN limed soil, liming increased the amount of Al in the shoots to near the threshold of hyperaccumulation (i.e., 1,000 mg Al kg^{-1}). This increase in shoot Al was a combined effect of the better growth, lower soil toxicity, and the presence of freely available Al^{3+}. This ability of *P*. sp. 'Frensham' to accumulate Al in the aboveground biomass had not been previously documented and this plant species may be added to the narrow list of Al accumulators. For the smelter-related metals, the similarities between the plants in metal uptake patterns was related to the extreme differences in the soils in terms of metal contamination, where physiological signs of toxicity (or even death) were observed in plants grown in the non-limed soils—decreasing the ability of the excluders to inhibit the translocation of metals into the shoots of the plants. Plant growth on the non-limed soils was also accompanied by high concentrations of LMWOA in the soil for the excluders—with the presence of specific LMWOA related to the natural acids found in the plant tissues. In this study, it was difficult to measure a significant connection between metal stress and the LMWOA in the soil due to (i) the extreme differences in plant survival and health on the non-limed soils compared to the limed soils, (ii) the different contaminants of concern on the SVN site (Zn, Cd, and Pb) compared to the KN site (Cu and Al), and (iii) sampling restrictions preventing the isolation of "rhizosphere" soil.

However, based on the r:s ratios and the extraction of the metals from the available fraction in the soil, differences were observed between the excluder and hyperaccumulator plants. Generally, *S. vulgaris* and *A. capillaris* (excluder plants) had higher r:s ratios for the heavy metals—when the plants were healthy—and removed a smaller fraction of the available metals from the soil into the shoots. For *P*. sp. 'Frensham' the shoot concentrations were higher and r:s ratios were lower, indicating that the plant was translocating a higher

fraction of the metals taken up to the shoots. Moreover, *P*. sp. 'Frensham' removed a higher proportion of the available metals from the soil, especially with Zn in the reference soil and Al in the KN limed soil where the plants were healthiest.

Overall, plant growth improved the conditions in all the soils, as increases in pH were seen for *S. vulgaris* and *A*. *capillaris* in the SVN soil, while all the plants increased the DOC in the soils and decreased the availability of certain metals in the soils. The only exception for this was for *A. capillaris* in the KN limed soil where the plant increased the available Cu^{2+}. However, as this soil had a low available Cu^{2+} concentration, this increase in available Cu^{2+} will likely not be problematic for plant growth or environmental contamination.

When selecting plants for revegetation of metal contaminated sites, problems exist in matching the metal tolerance indices with the actual conditions in the soil, especially for very large, highly variable, sites like in Flin Flon, MB/ Creighton, SK. As evident from this study, the native plant species *A*. *capillaris* performed the best in its native SVN soils, which were deemed non-responsive to the dolostone. Moreover, hyperaccumulation of metals from soil, and not hydroponic solutions, has really only been documented when plants are grown in native soils, even for *T. caerulescens,* correlating to the lack of true hyperaccumulation seen with *P*. sp. 'Frensham' (Roosens et al., 2003). Nevertheless, high concentrations of smelter-related metals and Al were still seen with *P*. sp. 'Frensham,' cautioning the use of hyperaccumulators in revegetation of low to moderately contaminated soils, as shoot uptake poses a threat to the continued transfer of heavy metals through the environment. In terms of revegetation, the same is true for excluders under metal stress evident by the concentration of metals in the shoots of *A. capillaris* in the KN non-limed soil. These higher shoot concentrations indicate that soil amendments, like lime, should still be used to decrease the uptake of heavy metals to the shoots of the plant, even if vegetation is present in the un-amended soil When revegetating heavy metal impacted sites, care needs to be taken to decrease both the mobility of the metals in the soil as well as the shoot concentrations in the plants.

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7 APPENDIX A

Fig. A.1. Site photographs from the reference area (Sherridon). (a) The vegetation consists of a stand of Jack pine with bearberry in the understory. (b) The soil for this site is an Eluviated Dystric Brunisol; depth to bedrock was >100 cm.

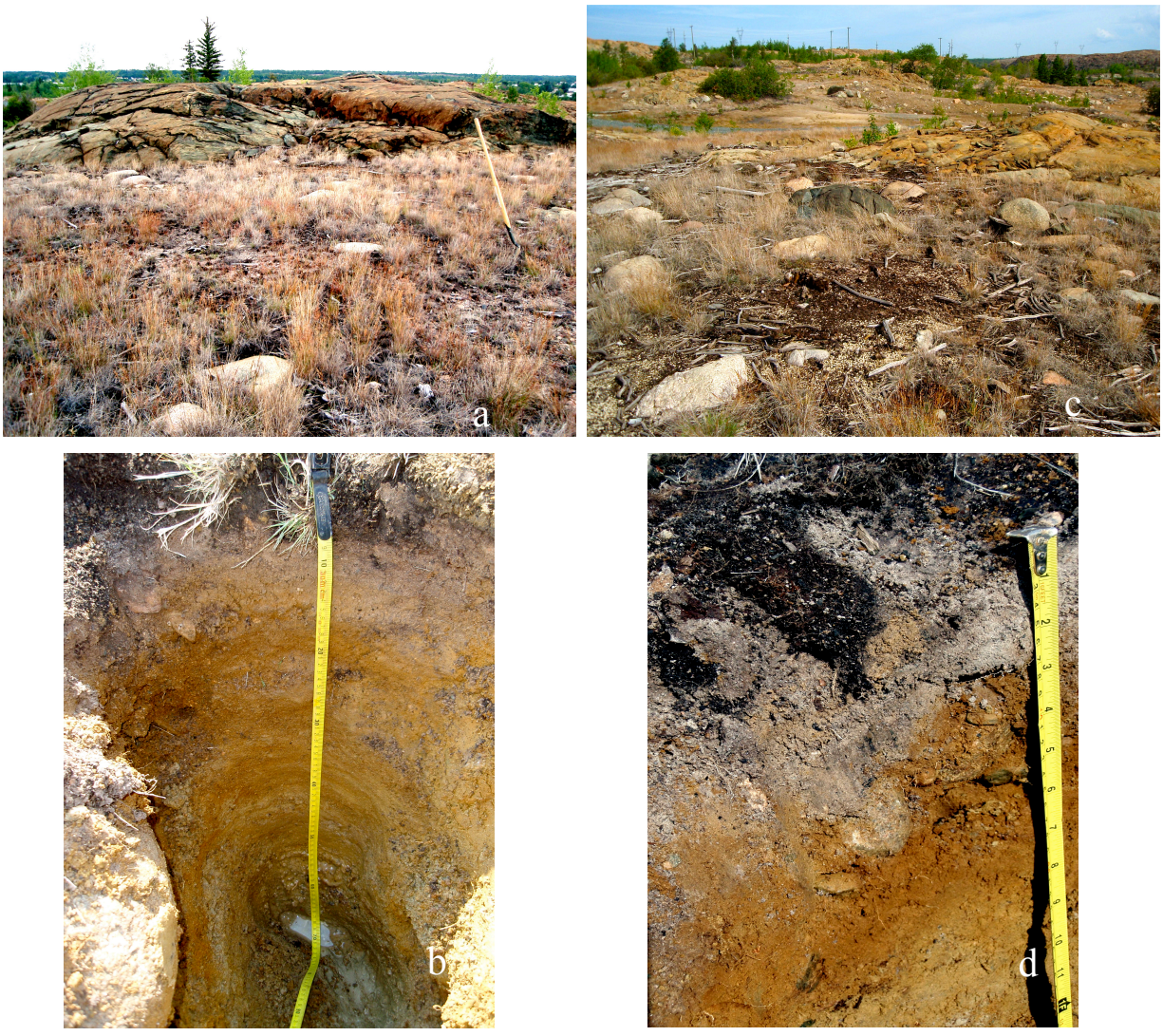


Fig. A.2. Site photographs from the Second Valley North site. (a and b) The vegetation and soil for the non-limed area consist of the stunted metal tolerant grass species *Agrostis capillaris* over an Eluviated Dystric Brunisol. (c and d) The vegetation and soil for the limed area also consist of the stunted metal tolerant grass species *Agrostis capillaris* over an Eluviated Dystric Brunisol; depth to bedrock was >100 cm.

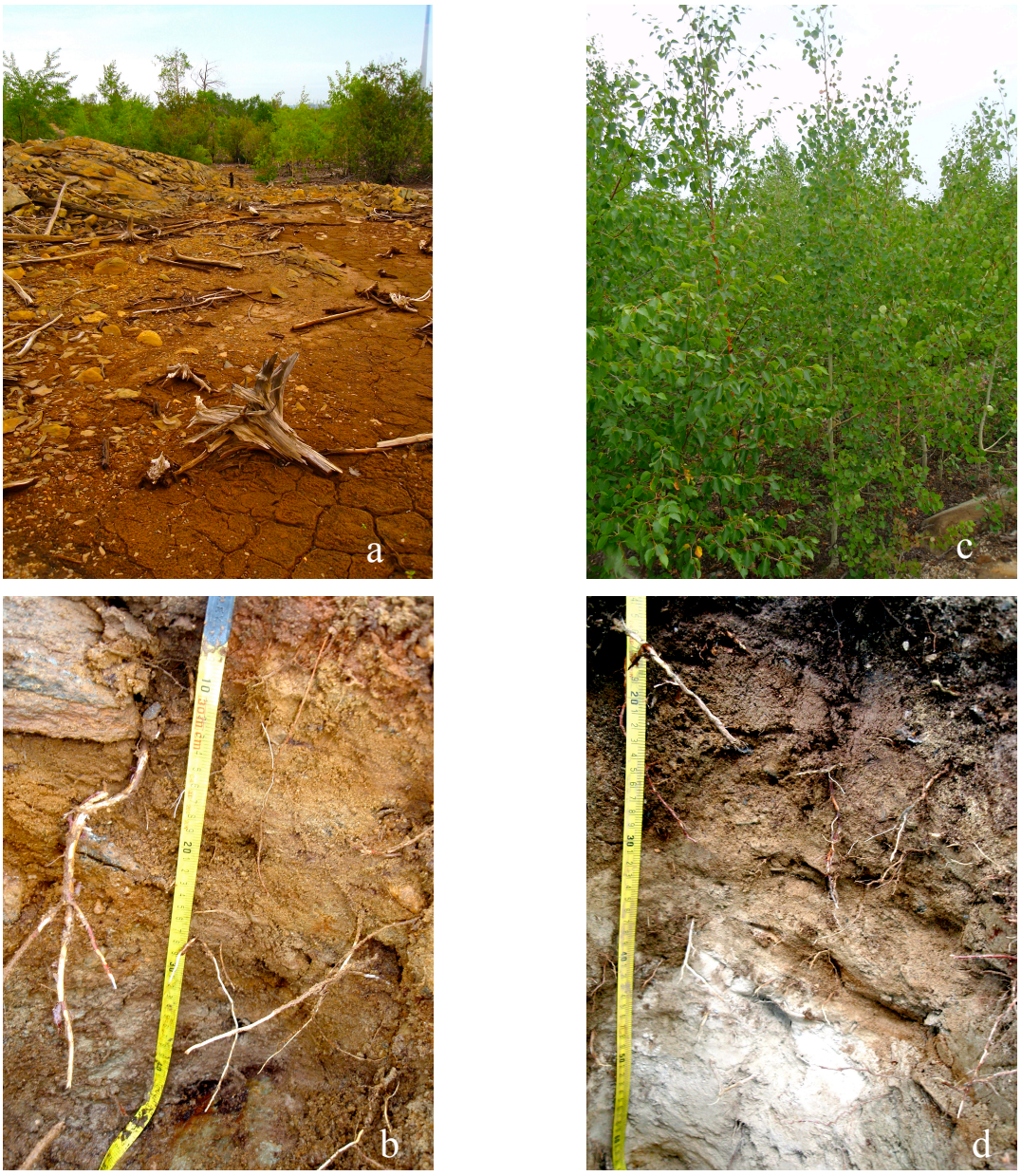


Fig. A.3. Site photographs from the Knight North site. (a and b) The landscape and soil for the non-limed area consist of barren, eroded areas over an Orthic Dystric Brunisol, depositional phase. (c and d) The vegetation and soil for the limed area consist of a healthy stand of birch and aspen with little to no understory over an Orthic Dystric Brunisol; depth to bedrock was generally <100 cm.

8 APPENDIX B

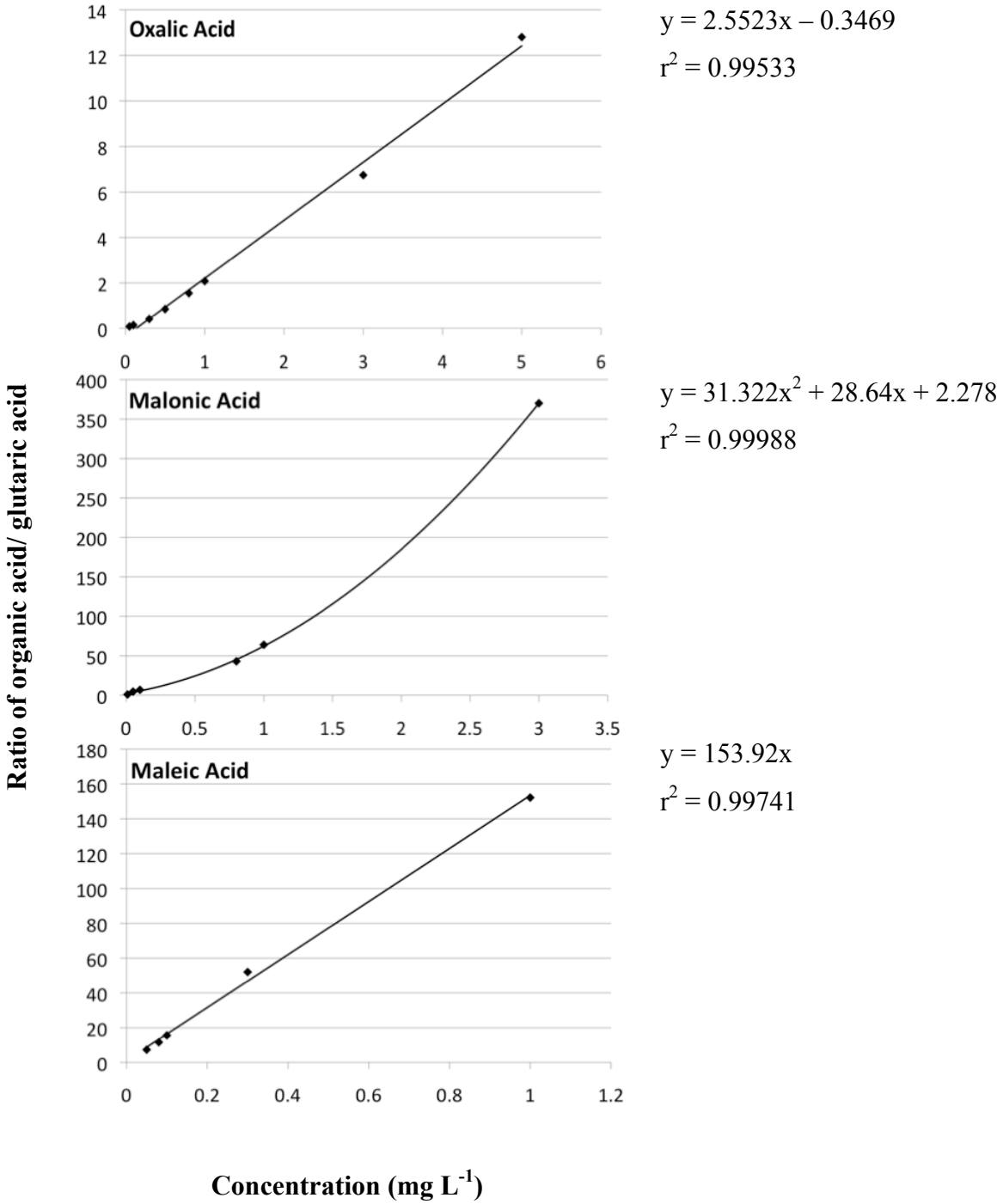


Fig. B.1. Calibration curves for oxalic, malonic, and maleic acid. The ratio of organic acid/ glutaric acid is in FID response (mV).

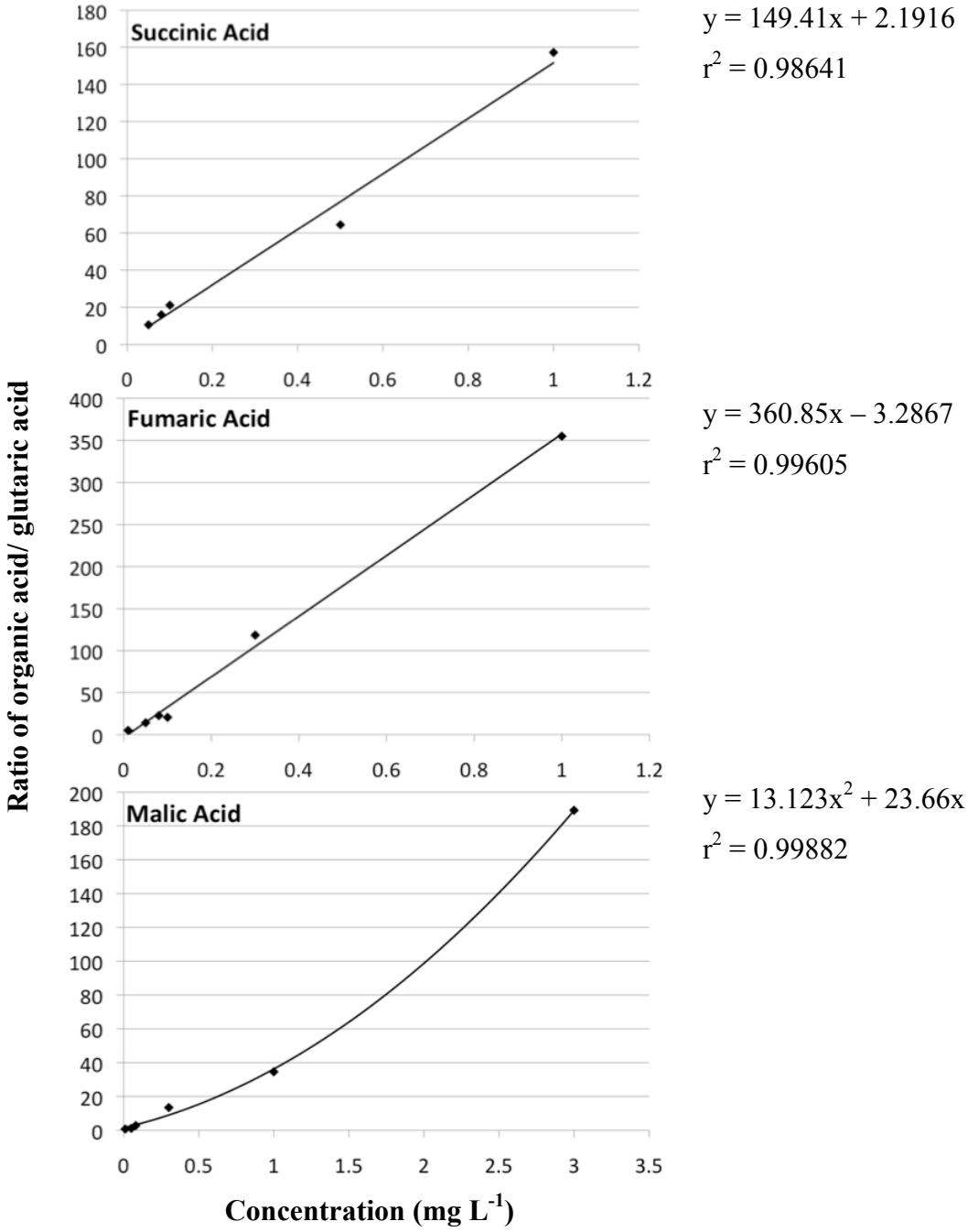
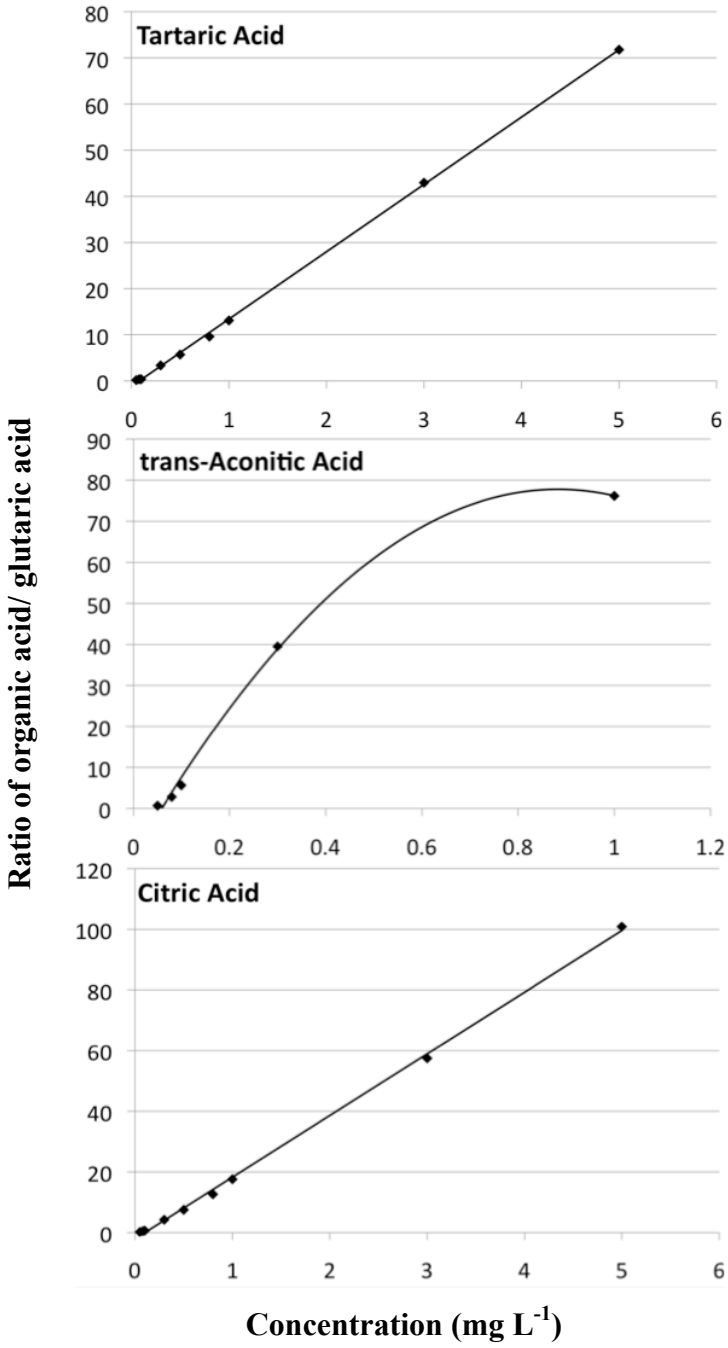


Fig. B.2. Calibration curves for succinic, fumaric, and malic acid. The ratio of organic acid/ glutaric acid is in FID response (mV).



$y = 14.59x - 1.1831$

$r^2 = 0.99964$

$y = -114.62x^2 + 202.4x - 11.5$

$r^2 = 0.99743$

$y = 20.331x - 2.0463$

$r^2 = 0.99895$

Fig. B.3. Calibration curves for tartaric, *trans*-aconitic, and citric acid. The ratio of organic acid/ glutaric acid is in FID response (mV).